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Exploring biological and pathological functions of
TGFβ family member activin C

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Zusammenfassung

Aktivine und ihre Antagonisten Inhibine sind Zytokine der Transforming Growth Factor (TGFβ) Familie, mit wichtigen regulatorischen Funktionen in verschiedensten physiologischen Prozessen. Aktivine sind Homo- oder Hetreodimere die aus zwei durch Disulphidbrücken verbundenen β Untereinheiten bestehen. In Säugetieren wurden bisher vier dieser Untereinheiten identifiziert: βA, βB, βC und βE. Im Gegenteil dazu bestehen Inhibine aus einer α und einer β Untereinheit. Während die βA mRNA und die βB mRNA in vielen Organen relativ hoch exprimiert werden, sind die βC und βE mRNAs hauptsächlich in der Leber vorhanden. Aktivin A ist das am besten erforschte Aktivin. Es steht im Zusammenhang mit Reproduktion, embryonaler Entwicklung, Homeostasis, Inflammation und Gewebereparatur. In der Leber leistet es einen Beitrag zur Regulation von Zellproliferation, Apoptosis und Gewebearchitektur. Zusätzlich zeichnet die Deregulierung von Aktivin A verantwortlich für pathologische Vorgänge wie hepatische Inflammation, Fibrose und Kanzerogenese. Die biologischen Funktionen der anderen Familien Mitglieder und deren Involvierung in Biologie und Erkrankungen der Leber sind bislang noch sehr unzureichend erforscht.

Der erste Teil dieser Arbeit beschäftigt sich mit der mRNA Expression der kompletten Inhibin Gen Familie, um neue Erkenntnisse über die möglichen Funktionen von Aktivinen und Inhibinen in humaner Hepatozellulärer Karzinogenese zu erlangen. Mit der Hilfe von quantitativer Real-Time PCR Analyse konnten wir eine starke Erhöhung der Inhibin α in hepatozellulären Karzinomen und umgebendem Gewebe im Vergleich zu Proben von gesunden Spendern sehen. Alle vier β Untereinheiten wurden in Proben von gesunden Spendern und von Patienten exprimiert, wobei die Expression der βB Untereinheit im Vergleich von normal zu maligne erhöht war. Diese Arbeit zeigt als erste einen signifikanten Zusammenhang zwischen den Expressionshöhen der Inhibin α und βB mRNA in humanen hepatuzellulären Karzinomen. Im Unterschied zu Daten aus verschiedenen Nagermodellen deuten diese Ergebnisse außerdem auf eine Tumor promovierende Rolle der Inhibine und Aktivine und deren entsprechenden Signalwege hin.

Wie auch andere TGFβ Familienmitglieder vermitteln Activin A und B ihre Aufgabe durch transmembrane Serin/Threonin Kinase Rezeptoren und Smad Proteine. Rezeptoren und nachfolgende Signalwege für Activin C und Aktivin E sind hingegen noch nicht sehr gut erforscht, wobei es Hinweise auf Smad unabhängige Mechanismen gibt. Im zweiten Teil der Arbeit ist es mir gelungen erstmals einen Unterschied in den Signalwegen von Aktivin C zwischen primären humanen Hepatozyten und Hepatomazelllinien zu zeigen. Aktivin C stimuliert Phosphorylierung von ERK in Zellen ohne basale Smad Phosphorylierung. Außerdem kann Aktivin C durch Phosphorylierung von ERK Apoptose induzieren, und zwar über einen Caspase 3 unabhängigen Mechanismus.

Wie bereits erwähnt ist Aktivin A in viele biologische Mechanismen involviert. Das Ziel des dritten Teils war es eine mögliche Rolle von Aktivin C in der embryonale Entwicklung, Apoptose, Inflammation und Kanzerogenese näher zu definieren. Wir konnten zeigen, daß Aktivin A und Aktivin C die Viabilität von Rattenembryonalzellen abhängig vom Tag ihrer Entwicklung, also abhängig von der Expression der jeweiligen Rezeptoren und somit Zugänglichkeit für die Faktoren, beeinflußt. Mittels eines gut etablierten Apoptose Zellmodells stellten wir eine modulierende Rolle für Aktivin C und die anderen Familienmitglieder in der Apoptose fest. Hinsichtlich Inflammation ist Aktivin C fähig basale wie auch IL6 induzierte T-Kininogen mRNA Werte in primären Rattenhepatozyten zu erniedrigen. Andererseits erhöht es die Sekretion von TNFα in LPS behandelten Kupffer Zellen. Gemeinsam mit den unterschiedlichen Effekten von Aktivin C auf die CRP Freisetzung in primären humanen Hepatozyten, die offensichtlich von der Anamnese der Patienten abhängen, betonen wir die Notwendigkeit eines vorangehenden Impulses für die Wirkung der Aktivine.

Zusammenfassend bestätigen die Ergebnisse dieser Studien die Rolle der Aktivine in physiologischen wie auch pathologischen Mechanismen, und zeigen eine modulierende Rolle für Aktivin C. Die hier vorliegende Identifizierung eines Smad unabhängigen Signalwegs für Aktivin C ist ein wichtiger Schritt für die zukünftige Aufklärung der stark verflochtenen Aufgabengebiete dieser biologisch sehr komplexen TGFβ Familienmitglieder.

Abstract

Activins and their antagonists inhibins are cytokines of the transforming growth factor β family (TGFβ), with important regulatory functions in a wide array of physiological processes. Activins are homo- or heterodimers consisting of two disulfide-linked β subunits, four mammalian activin β subunits - βA, βB, βC, βE - have been identified in mammalian cells. Inhibins in contrast are heterodimers composed of an α subunit and a β subunit. Whereas the expression of βA and βB subunits is high and widely distributed in many organs, the βC and βE subunits are predominantly expressed in the liver. Activin A is by far the best investigated activin. It has been implicated for instance in reproductive biology, embryonic development, homeostasis, inflammation and tissue repair. In the liver it contributes to regulation of cell growth, apoptosis and tissue architecture. Additionally, deregulation of activin A signaling accounts for pathologic conditions such as hepatic inflammation, fibrosis and carcinogenesis. The biological functions of the other family members and their involvement in liver biology and diseases are still poorly understood.

The first part of this work deals with the mRNA expression pattern of the complete inhibin gene family to obtain novel insights into possible functions of activins and inhibins in human hepatocellular carcinogenesis. Using quantitative real-time PCR analysis we found strongly increased inhibin α subunit expression comparing samples of hepatocellular carcinoma and tumor surrounding tissue to samples from healthy donors. All four β subunits were expressed in normal and patient samples, whereas expression of βB subunit increased from normal to malignant samples. This study is the first to report a significant relation of the inhibin α and inhibin βB mRNA levels to human hepatocellular carcinoma. Furthermore, these data, different from those in rodent model systems, suggest a tumor promoting role of inhibin and activin proteins and their respective pathway.

Like other members of the TGFβ family activin A and B are known to elicit their actions through transmembrane serine/threonine kinase receptors and Smad proteins. Receptors and downstream signaling for activin C or E however are hardly elucidated, whereas there are data suggesting Smad independent mechanisms. In the second part of this work we show for the first time that activin C signaling differs between primary human hepatocytes and hepatoma cell lines, as activin C stimulates phosphorylation of ERK in cells without basal Smad phosphorylation. Furthermore we report that activin C is able to induce apoptosis by ERK phosphorylation in a Caspase 3 independent way.

As mentioned above activin A is known to be involved in many biological mechanisms, like development, apoptosis, inflammation and cancerogenicity. The objective of the third part was to define the role of activin C in these biological functions. Thus, we report that activin A and C effect viability during development depending on the day of growth, hence expression of the respective receptor and accessibility for these factors. Data gained on the basis of a

well established apoptosis cell model suggest a modulatory role for activin C and the other activin subfamily members regarding apoptosis. With respect to inflammation activin C reduces basal as well as the IL6 induced T-kininogen mRNA levels in primary rat hepatocytes, whereas it enhanced the release of TNFα in LPS treated Kupffer cells. Together with the differential effects of activin C on CRP release seen in human primary hepatocytes, apparently depending on the anamnesis of patients, we emphasize the importance of a trigger necessary to induce the properties of activins.

These studies demonstrate the involvement of activins in many physiological but also pathological mechanisms, and show that activin C modulates basic biochemical pathways. The present identification of one possible signaling mechanism for activin C is an important step to elucidate the interwoven fields of action of these very potent TGFβ family members in the future.

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I. Introduction

1. Protein and Gene Identification

Activins and inhibins are members of the mammalian transforming growth factor-β (TGFβ) family, involved in regulation of embryogenesis, organogenesis, reproductive processes, inflammation, homeostasis and cancerogenesis (Chang et al., 2001). The superfamily itself comprises more than 35 members in vertebrates, including TGFβs, BMPs (bone morphogenic proteins), GDFs (growth differentiation factors), activins, inhibins, MIS (Müllerian inhibiting substances), Nodal and leftys (Dube et al., 1998; Hogan, 1996; Kingsley, 1994). Existence of a gonadal negative feedback regulator of pituitary function has been postulated 1923 (Mottram et al.), and it was termed inhibin by McCullagh in 1932. He isolated inhibin from bovine testis where it inhibited hypertrophy of pituitary cells. As a gonadal hormone it is part of a feedback loop exerting negative action on the release of follicle stimulating hormone (FSH). With the purification and characterization of inhibin from various species, two fractions with inhibin-like activity were isolated. The larger protein with a molecular mass between 40 and 70 kDa was found in gonadal extracts and fluids, the smaller with molecular mass between 5 and 20 kDa was found in seminal plasma (reviewed by De Jong et al. 1985). Inhibin isolated from porcine follicular fluid was shown to be a 32 kDa heterodimeric protein, the two fractions where characterized as subunits and were named α and β chain (Mason et al. 1985).

At this time another protein consisting of two β subunits was isolated. It was found to stimulate FSH release, thereby antagonizing the action of inhibin and thus was termed activin (Ying et al. 1986). Studies first revealed two different β subunits, namely βA and βB (Ling et al. 1986, Vale et al. 1986). Another β subunit called βC was then identified from a liver cDNA library in human (Hoetten et al. 1995) followed by mouse (Lau et al. 1996) and rat (Fang et al. 1996). Soon after that, the subunit βE was cloned from liver transcripts in mouse, rat, and human (Fang et al. 1996, O'Bryan et al. 2000, Hashimoto et al. 2002). Interestingly, the subunits differ in their expression pattern as βA and βB mRNA are found ubiquitously (Tuuri et al 1994, Vejda et al. 2002), but βC an βE are predominantly expressed in the liver (Vejda et al. 2002, Hashimoto et al. 2002, Loveland et al. 1996, Gold et al. 2004, Thomas et al. 1998, Mellor et al. 2000). Beside these mammalian subunits, a more distantly related subunit has been identified in Xenopus named βD (Oda et al. 1995).

Like most TGFβ family members, activins and inhibins are dimeric entities and the two subunits are linked via disulfide bonds. With respect to the cellular expression level of the different subunits several variants of activins/inhibins are possible. On the one hand there are the inhibins A (αβA) and B (αβB) that have been isolated from porcine follicular fluid

(Vale et al. 1986), and inhibin C (αβC), who's existence so far was only described after transfecting human activin C expressing CHO cells with inhibin α (Ushiro et al. 2006). On the other hand there are the activin homodimers that are composed of two identical β-subunits, of which activin A is the most comprehensively studied so far. Furthermore heterodimers consisting of different β-subunits are formed, for example activin AB or AC (Vejda et al. 2002, Ling et al. 1986, Mellor et al. 2003, Nakamura et al. 1992).

2. Gene Structure

The five subunits that assemble the different kinds of activins and inhibins are encoded by different genes located on different chromosomes (Table 1). They are grouped within the inhibin subfamily and are named InhβA for the α-subunits gene, and IbhβA, InhβB, InhβC and InhβE for the respective β-subunit genes.

The genomic organization is relatively consistent, as all their coding regions are distributed on 2 exons separated by an intron, ranging from 0.2 to 14 kb. IbhβA displays the only exception as it encloses a third exon upstream of exon 1 encoding for a 5'untranslated region (Tanimoto et al., 1996).

Human InhβA and InhβB are both located at chromosome 2, while InhβA resides at the proximal region of chromosome 7. The mouse βC and βE genomic loci are determined to be at the distal region of chromosome 10, while the human genes maps to the long arm of chromosome 12. Analyses revealed the activin βC and βE genetic loci to be closely linked, thus their coding sequences are only separated by 5.5 kbp. The murine genes of activin βC and βE span approximately 14kb and are similarly organized. Like in other TGFβ subfamily members genes each consists of two exons separated by a single intron. Exon 1 in both cases encodes for amino acids 1 to 106, which comprise the signal peptide and amino-terminal portion of the propeptide domain. Exon 2 on the other hand codes for the remainder of the propeptide domain and the entire mature domain. The activin βC intron is the size of 11.5 kbp, the activin βE intron in contrast consists of 236bp (Fang et al., 1997; Schmitt et al., 1996). In other species these two TGFβ family members are located in proximity on the same chromosome too as reviewed by (Grusch et al., 2007). Both genes show a closely linked location and similar expression patterns restricted primarily to the liver, which is why it is suggested that they were generated by tandem duplication of an ancestral gene (Fang et al., 1997).

Gene	Chromosome	Exon-Intron	Literature
βA (InhbA)	7p15-p13	3 exons, 2 and 9.5kb intron	Tanimoto 1996
βB (InhbB)	2cen-q13	2 exons, 2.4kb intron	Mason 1989
βC (InhbC)	12q13.1	2 exons, 14kb intron	Schmitt 1996
βE (InhbE)	12q13.3	2 exons, 0.2kb intron	
α (InhA)	2q33-q36	2 exons, 2kb intron	Stewart 1986

Table 1. Human activin/inhibin subunits (according to Grusch et al., 2007)

3. Protein structure

The basic structures of the activin peptides very much resemble each other, as well as that of other TGFβ family members. They are translated as prepropeptides, made up by the typical hydrophobic signal peptide, followed by the prodomain with its potential N-linked glycosylation sites, and the C-terminal mature peptide. In all peptides a stretch of basic amino acids precedes the predicted proteolytic cleavage site and all contain the nine cysteins that form the cysteine knot characteristic of TGFβ-family peptides (Hogan, 1996; Massague, 1998; Padgett et al., 1997; Vejda et al., 2002b). The precursors are composed of 350-426 amino acids, which results in a molecular weight between 38 and 50kDA. Their respective prodomain is removed to release mature peptides ranging from 112 to 134 amino acids.
Sequence identity is on average 30-50% for proteins in different subgroups and 60-80% for proteins in the same subgroup (Griffith et al., 1996). Mouse βC shows 53% amino acid identity to the mature βB subunit and 51% to the mature activin βA subunit, whereas βA and βB show an identity of 64% to each other (G et al., 1995). Mouse βC and βE in turn are highly homologues, with 61% amino acid identity over the mature growth factor region (Fang et al., 1996). Comparison of all four human β-subunits revealed the mature peptides to be more conserved than the prodomains, which only show a sequence homology about 20% (Grusch et al., 2007). Phylogenetic analyses suggest, that βA and βB are most related to each other while βC and βE represent a second subset of related sequences. This applies not only for comparison of the mature peptide but also for comparison of the prodomain.

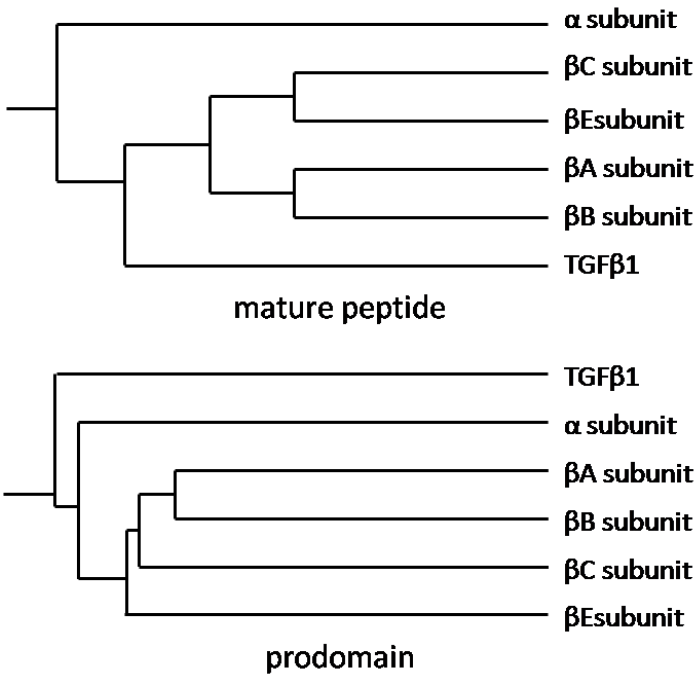

Fig.1 Phylogenetic trees of amino acid sequence of mature peptide and prodomain, comparing human activin respectively inhibin subunits and TGFβ1 according to Grusch et al.

3.1. Prodomain

The prodomain of TGFβ family members determines the folding, dimerisation and export of the respective mature cytokine (Gray and Mason, 1990). TGFβs are secreted as biological latent forms. The activity of the mature domain of the TGFβ dimer is masked by its prodomain, also termed latency-associated propeptide (**LAP**), which is cleaved from the mature protein by a furin-like endoprotease during secretion but remains associated with it via noncovalent interactions (Dubois et al., 1995). This so called small latent complex is disulphide linked to a single molecule of latent TGFβ binding protein (**LTBP**) now forming the large latent complex.

LTBP themselves are large ECM proteins, structurally related to fibrillins. These binding proteins therefore do not only play a regulatory role as modulators of TGFβs availability, but also a structural one as components of matrix. Complex formation is mediated by intramolecular disulfide exchange between the third 8-cysteine domain of LTBP with Cys-33 in the N-terminal region of LAP (Gleizes et al., 1996; Saharinen et al., 1996). Brunner et al. reported that loss of this cysteine bridge leads to enhanced secretion of bioactive TGF-β1 dimer (Brunner et al., 1989). Mutation of one cysteine in the CxC-motif at the C-terminal region of LAP has no effect, mutation of both, in contrast, prevents LAP dimerisation but has no effects on the TGF-β1 bioactivity. In the βA prodomain mutation of all four cystein

residues where shown to neither alter intracellular processing, dimer assembly, nor secretion (Mason, 1994). Grusch et al. report a different cysteine distribution for βC and βE in their prodomain compared to that of βA. They also found a classical nuclear localization signal in the βA and βE prodomain, as well as a leucine zipper, which represents a dimerisation motif, in the prodomain of βC. Furthermore a WHxL motif was detected in the prodomain of both human βC and βE subunits, but only in βE in mouse and rat (Grusch et al., 2007). The N-terminus of βB prodomain contains an unusual proline-rich region which often are dimerisation structures and moreover are interpreteted as Src-Homology3 (SH3) ligand regions (Mason, 1994).

The TGFβ prodomain contains three potential asparagine-linked glycosylation sites. The first and the second N-linked carbohydrates contain mannose-6-phosphate and stabilize the mature TGF-β dimer in the latent complex. N-linked glycosylation is necessary for translocation to the Golgi apparatus and mutation of these sites decreases secretion of the mature TGF-β1 dimer (Brunner et al., 1992). The βA, βB and βE prodomains similar to the α-subunit contain one N-linked glycosylation site, whereas βC like LAP exhibits three potential N-linked glycosylation sites (G et al., 1995).

All in all the prodomain is poorly conserved across the different activin family members as it is the case for other subfamily members of the TGFβ superfamily.

3.2. Mature protein

Cystein knot

The TGFβ family members are all characterized by a distinct structural feature, namely a cysteine knot scaffold. The three dimensional structure resulting from the cysteine knot folding of the proteins is responsible for several features. Function as extracellular protein, interaction with specific receptors and other extracellular proteins are just some of them (Vitt et al., 2001).

Three distinct domains are formed due to the special folding: In each activin monomer two pairs of antiparallel β strands form so called finger-like projections that stretch out from the cysteine core of the dimer. The characteristic curvature of the fingers creates concave and convex surfaces on the butterfly-shaped ligand. The base of the fingers is formed by an α-helix together with a preceding loop and is also known as the wrist epitope (Greenwald et al., 2004; Thompson et al., 2003). Therefore, the cysteine knot forces the protein into a three-dimensional structure that exposes hydrophobic residues. This exposure is the precondition for forming homo- or heterodimers (Sun and Davies, 1995). Additional cysteine residues strengthen the dimerisation by forming covalent disulfide bridges between the subunits of the dimers (Hui et al., 1999; Prestrelski et al., 1994).

TGFβ and activin β subunits share 9 conserved cysteines, whereas inhibin α subunits contain only 7 cysteine residues. In vitro mutagenesis studies revealed that in contrast to the four cysteine residues in the proregion of activin A precursor, which are not necessary for biosynthesis of mature activin A, the nine cysteines in the mature form are all important for biosynthesis and biological activity. Cystein residue 80 is disulfide linked to the corresponding residue in the second subunit of the activin dimer, thus mutations in this residue leads to expression of an activin monomer. Furthermore this cysteine is involved in dimer stabilization via hydrophobic interactions with a valin at position 82. The cysteine residues 4 and 12 are not needed for dimerisation because the intramolecular disulfide bond between them is not involved in the cysteine knot. In fact, these two residues are important for the biological function since their removal results in a 2-fold reduction in biological activity and a 3-fold reduction in receptor affinity. Mutations in cysteine residues 44 and 113 lead to secretion of proteins with lower respectively no bioactivity, whereas the remaining activin cysteine mutants failed to be secreted at all (Mason, 1994).

Receptor binding sites

As discussed later in detail, activin signaling involves binding of the dimer to type I and type II transmembrane serine/threonine kinase receptors. First activin binds to its constitutively active type II receptor kinase (ActRII/ActRIIB), allowing the recruitment, phosphorylation and activation of the type I receptor (Alk4) (Lebrun and Vale, 1997; Mathews, 1994).

The crystal structure of the **ActivinA:ActRIIB** complex illustrates that the ActRIIB makes contact with the convex outside of the finger region of its ligand, at which each of the monomeric subunits of the ligand interacts with one type II ectodomain (Thompson et al., 2003). The interface involves hydrophobic and ionic/polar residues in finger 1 and finger 2 of the activin (Thompson et al., 2003). These residues are conserved within the activins, presuming a similar binding of the isoforms to the type II receptor (Thompson et al., 2004).

As less is known about the **activin:ALK4** interface, predictions had to be made based on the crystal structure of the BMP2:Alk3 complex. Type I receptor interaction takes place on the concave side of the finger of one subunit, on the opposite side of the type II receptor binding site, and on the α-helix of the other subunit of the dimer. Almost every hydrophobic residue at the core of the interface is conserved between activin A and B, indicating similar affinities of the two isoforms to the receptor (Thompson et al., 2004). Activin A and activin C are known to share only 2 of 13 residues of the α-helix, whereas no reference data for the activin C finger structures are available.

Most mutants with changes within the prehelix loop (Pro^{45}-Ser^{57}) and the α-helix had little effect on activin A activity. Only activin A with substantial mutations such as exchanging the

entire α-helix for that of activin C, or inserting proline residues at certain points, were unable to confer activity. This indicates that the α-helix indeed is important for activin A function, but binding is not dependent on integrity of the helix structure (Harrison et al., 2004).

Follistatin binding sites

Binding sites for the most important antagonist of activin signal transduction, follistatin, which is described later, are well characterized. Follistatin contacts two neighboring surfaces on activin A (Keutmann et al., 2004). One surface overlaps the type II receptor binding site, including residues on the convex outer site of the β-strands and on the fingertips. The second binding site includes the proposed type I binding surface, and is formed by residues of the concave finger site of one subunit and the wrist region of the second subunit. Mutagenesis studies showed that especially Leu^{92}, Tyr^{94}, Ile^{100} and Lys^{102} are important for high affinity binding of follistatin, whereas mutations of Ser^{90}, another important residue for ActRII binding, did not affect it, indicating the interaction surfaces for type II receptor and follistatin to be overlapping but not identical (Harrison et al., 2006).

Alpha-helix

The helix forms the dimer interface, whereas in activins it would putatively consist of residues 58-69. Interestingly activins have more flexible residues in the helix and the corresponding loop than other TGFβ ligands, which may contribute to the position and stabilization of the dimers. The conformational flexibility may also play a key role in adopting folded back confirmations for simplified receptor recruitment by bringing them into closer proximity (Thompson et al., 2004)

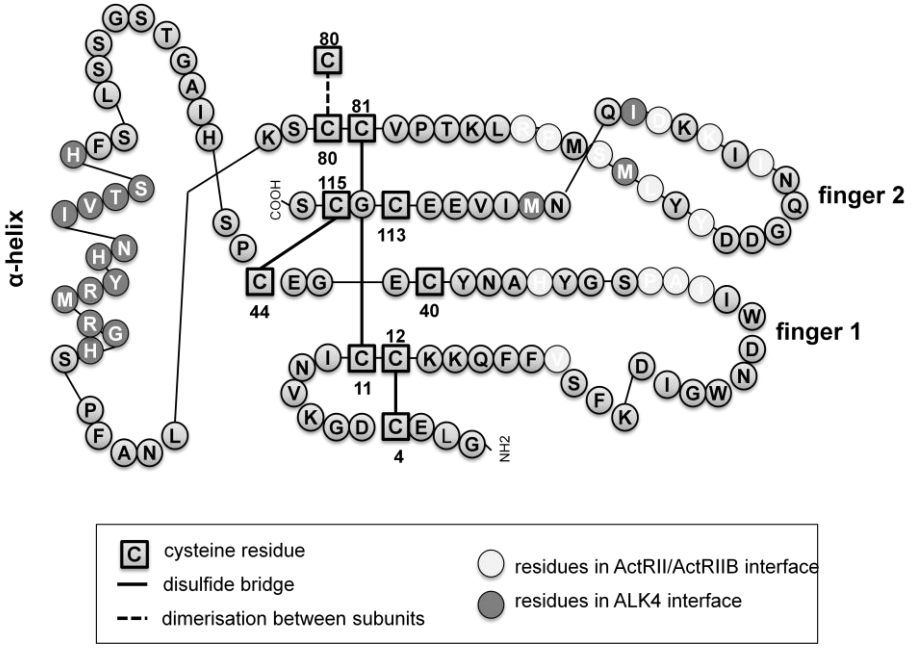

Fig.3 Schematic diagram of the human Activin A monomer (according to Harrison et al., 2006; Mason, 1994)

4. Processing and Secretion

Very little is known about the intracellular assembly, processing and secretion of inhibin and activin, but as TGFβ family members are translated as inactive proteins, proteolytic processing of the prepropeptides is an essential step in the formation of the mature biologically active homo- or heterodimers.

On ribosomes newly synthesized proteins are targeted to the endoplasmatic reticulum protein channels by a hydrophobic signal peptide and are inserted into the lumen during synthesis. This compartment is the location of protein folding and dimerisation, thus proteins gain their correct three dimensional structures during their transit through it (Murshid and Presley, 2004). Antenos et al. showed that for inhibin α and βA subunits an early step in this process involves the attachment of N-linked oligosaccharides to asparagines residues in the sequence Asn-X-Ser/Thr in the polypeptide chain (Antenos et al., 2007). This directs the differential assembly of inhibin and activin dimers, meaning that the presence or absence of a specific glycosylation site can influence whether inhibin or activin is produced, and also their secretion and stability (Antenos et al., 2007). Known enzymes that catalyze all the reactions of native disulphide bond formation in the ER are the **protein disulphide isomerases** (Ellgaard and Ruddock, 2005). This member of the thioredox family can catalyze thiol-disulphide oxidation, reduction and isomerization, the last of which occurs

through intramolecular disulphide rearrangements or through cycles of reduction and oxidation (Ferrari and Soling, 1999; Schwaller et al., 2003). Proper folding is the precondition for further translocation to the Golgi network and for secretion. In the Golgi apparatus other posttranslational modifications occur including cleavage of the prodomain thus permitting release of the mature peptide. Involved enzymes are the **family of subtilase—like proprotein convertases** (Salvas et al., 2005). SPC 2 and 3 are predominantly expressed in neuroendocrine tissue, whereas SPC1 (also named furine) and SPC4 are ubiquitousily expressed, with highest levels in the liver. Furin, like all other SPCs, is calcium dependent, furthermore has a neutral pH optimum and is localized predominantly to the trans golgi network where it is retained by a c-terminal transmembrane domain (Rouille et al., 1995). It is responsible for the maturation of several members of the TGFβ family, including the inhibin α and βA subunit, all containing the RXXR recognition motif conserved in their precursor protein (Dubois et al., 1995; Molloy et al., 1999). It has to be noted, that cleavage of activin A precursor (pro-βA) is necessary for the release of bioactive mature ligand (βA), whereas retention of the α subunit precursor (pro-αN-αC) has no influence on the biological activity on the inhibin molecule, when the β-subunit can be normally expressed (Mason et al., 1996). The βB subunit in contrast to βA does not contain the exact furin cleavage site, however, its recognition motif IRKR is sufficient for processing (Nakayama, 1997). Grusch et al. report a RXRR recognition motif for the βE subunit too, as well as suitability for cleavage through SPC7. The cleavage motif in βC is optimal for nardilysin or SPC2/3 (Grusch et al., 2007). Furin is not only the key enzyme in cleavage and maturation of the inhibin α and InhβB subunit, but is also part of a feedback loop regulating the secretion of bioactive activin B and inhibin B. Activin A positively regulates furin mRNA levels, which in turn lead to an increase in both inhibin α and activin βB subunit protein maturation and secretion (Antenos et al., 2008).

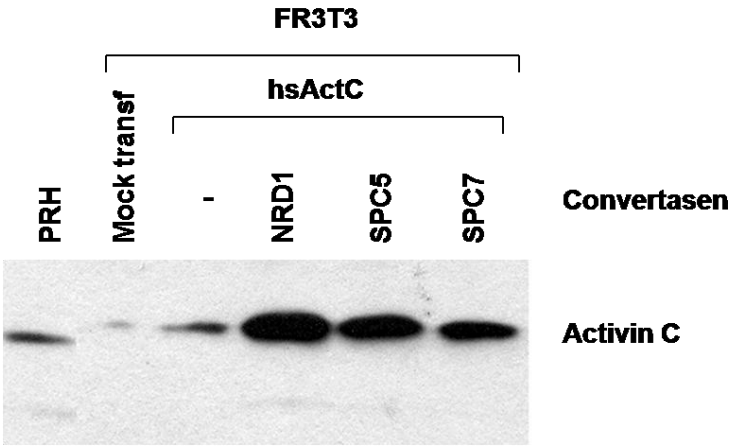

Fig.4 Enhancement of activin C protein expression through convertases.
FR3T3 were transiently transfected with activin C together with the respective convertases. Total lysates were separated with SDS-PAGE and transferred to PVDF membranes. Detection was performed using the S2 antibody kindely provided by Wolfgang Schneider (Department of Medical Biochemistry, Max F. Perutz Laboratories, Medical University of Vienna). As reported before, nardilysin is the optimal convertase for activin C. Also SPC5, which served as control and SPC7, which is the optimal convertase for activin E enhanced secretion of activin C. This enhancement of protein expression with the help of different convertases reflects sequence similarities between the different activins, but also relatively low specificity of the convertases.

5. Activation

After dimerisation and proteolytically processing of the TGFβ family members via furin-like proteases, the thereby gained mature peptide and the N-terminal propeptide part remain noncovalently bound. This aforementioned small latency complex is associated covalently to LTBP forming the large latent TGFβ, which is secreted by most cells. Latent TGFβ cannot bind to their receptor and therefore need to be released from the latent complex by activation.

This can be achieved on the one hand by physicochemical activation, like changes of the pH, treatment with chaotropic agents like urea or SDS, as well as by increasing the temperature (Brown et al., 1990). Also the removal of the glycosylation of distinct residues in the LAP peptide has been reported to activate TGFβ. Enzymatic removal of the sugar structures results in conformational changes which in turn disables the LAP to further mask the mature TGFβ resulting in its activation (Miyazono and Heldin, 1989). Also events that increase reactive oxygen species in the extracellular space like exposure to irradiation may lead to changes in the structure of latent TGFβ complex and thereby release of the mature protein (Barcellos-Hoff and Dix, 1996).

On the other hand activation can be mediated by proteinases. Plasmin for example, dissociates the latent complex by proteolytic cleavage (Lyons et al., 1988). Thrombospondin (TSP1) can activate TGFβ by inducing conformational changes in the latent complex (Murphy-Ullrich and Poczatek, 2000).
Another mechanism of activation, resulting in conformational changes again, is interaction of RGD sequences on the LAP with the integrin αVβ6 located on epithelial cells. In contrast to other conformational changes, there is no release of LAP, nevertheless the mature protein is able to interact with the type II receptor(Khalil, 1999).
The TGFβ family's composition of many different members, as well as it's involved in many physiological processes in almost all tissues, suggests that proteins as complex are activated by a mixture of several different mechanisms rather than having just one single universal way of activation.

6. Receptors

Receptor mediated signal transduction involves ligand dependent activation of receptors, whose intracellular domains control the recruitment and activation of mediators of the intracellular signaling pathway. Consequently, the first step in the TGFβ signaling cascade is the assembly of the dimeric ligand and two pairs of type I and type II receptor serine/threonine kinases on the cell surface.
The receptor serine/threonine kinase family in the human genome comprises 12 members: 7 type I and 5 type II receptor. Both types of receptors consist of about 500 amino acids, forming a small N-terminal extracellular ligand binding domain, a single transmembrane segment, and a C-terminal cytoplasmatic serine/threonine kinase domain. The characteristic structural feature of the TGFβ family of receptors is the three-finger toxin fold in the ligand binding extracellular domain, with each finger formed by a pair of anti-parallel β strands (Shi and Massague, 2003).
It has been shown that ligands activate the two types of receptors in a cooperative manner, such that the lower affinity receptor is recruited only after the ligand is bound to the other ligand. In the case of activin and also TGFβ itself, the type I receptor is the lower affinity receptor, thus it cannot bind the ligand in absence of the type II receptor (Wrana et al., 1994). The type II receptor very well binds ligands in absence of type I receptors, however is not able to signal in the absence of the latter (Wrana et al., 1992). In the receptor complex, the constitutively active type II receptor, which is ActRII/ActRIIB (Activin receptor II/IIB) in case of activin A, phosphorylates the receptor type I ALK 4 (Activin like kinase 4) in a highly conserved 30 amino acid serine/glycine rich segment (GS domain). This phosphorylation is

associated with conformational changes within the GS box, enabling ATP binding and phosphorylation of the cytoplamatic Smad2/3, which are part of the post-receptor signal transduction system (Attisano et al., 1996; Derynck et al., 1998). In contrast to activin A activin B and AB have a preference for ALK7 as type I receptor (Tsuchida et al., 2004). Receptors for activin C and E have not been reported so far. However, activin C is known not to interfere with the binding of activin A to its receptor (Wada et al., 2004b). A chimeric activin construct, in which the activin A receptor binding sequence (amino acids 46-78) was replaced by the corresponding sequence of βC, still was able to bind to ActRII, but was unable to recruit Alk4 (Muenster et al., 2005).

7. Signaling

Smad proteins are the TGFβ family receptor substrates that further transduce signals to the nucleus and to the target genes. There are three functional groups of Smad proteins: the receptor regulated Smads (R-Smads), the Co-mediator Smads (Co-Smads) and the inhibitory Smads (I-Smads). The first group consists of Smad 1, Smad 5 and Smad 8 which are phosphorylated by BMP type I receptors, and Smad 2 and Smad 3 that are activated by TGFβ and Activin type I receptors. Only one Co-Smad is known so far, namely Smad 4, and thus is a shared component in signaling of all TGFβ family members. The I-Smads comprise Smad6 and Smad7, inhibiting signaling by disabling activation of R- and Co-Smads.

The latter two share sequence similarities in two conserved globular domains at their N- and C-termini, termed MH1 and MH2 domain (Mad homology 1 and 2) which are connected by a proline rich linker region. The function of MH1 is to recognize the DNA sequence CAGAC and it may play a role in nuclear import and as negative regulator of the MH2 domain. The N-terminal domain of I-Smads displays only weak sequence homology to the MH1 domain, while the MH2 domain is highly conserved among all Smad proteins. It is the site of receptor interaction, binds the transcriptional coactivators and corepressors, is responsible for formation of Smad complexes and directly contacting of the nuclear pore complex. The highly divergent linker sequence contains multiple phosphorylation sites, which allow crosstalk with other signaling pathways, and a PY motif that is the interaction site for Smurfs. Smurfs in turn are E3 ubiquitin ligases that target Smads for degradation by proteasomes, and together with dephosphorylation play a role in termination of the signaling (Itoh et al., 2000; Massague et al., 2000; Shi and Massague, 2003).

To ensure the availability of Smad 2 and 3, they are kept in the cytoplasm by binding to Smad anchor for receptor activation (SARA). This interaction also assures the presentation to the receptor for phosphorylation (Tsukazaki et al., 1998). The binding sites between

receptor and R-Smad are made up of the GS region on the one hand and the L3 loop in the MH2 domain on the other hand (Lo et al., 1998). Upon aforementioned receptor phosphorylation, which occurs at the C-terminal SXS motif, the affinity of Smad2/3 for SARA is decreased, resulting in the dissociation of the receptor/activin/SARA complex, and exposure of the nuclear import region. Simultaneously phosphorylation increases the affinity of the R-Smads for Smad4, whereas their association is important for entrance to the nucleus (Xu et al., 2000).

Smad 4 and all R-Smads except Smad2 bind to a specific DNA sequence (5'-AGAC-3'), termed minimal Smad binding element (SBE) (Dennler et al., 1998; Jonk et al., 1998; Zawel et al., 1998). However, binding to SBE alone is not sufficient for Smad-dependent transcriptional activation. This is, on the one hand due to a very low affinity of the MH1 domain for SEB, which is not sufficient for effective binding (Shi et al., 1998). On the other hand Smad binding to the SBE lacks selectivity, because all the R-Smads and also Smad4 share the same β-hairpin sequence. Thus, so called transcription factors, which are described later on, are necessary to provide a tight target gene specificity (Attisano and Wrana, 2000; Massague and Wotton, 2000).

7.1. Smad independent signaling

When considering the diversity of responses mediated by just a few receptor-ligand combinations, it seems likely that the TGFβ family activates other pathways than the canonical Smad one too. In fact, several reports suggest cell type- and condition-dependent regulation of alternative signaling pathways including MAP Kinases like ERK, JNK and p38, but also Rho-like GTPase signaling pathways and PI3/Akt pathways. These pathways may be simultaneously activated with the Smad pathway to converge on the target genes resulting in an enhancement of the Smad-dependent response, some may regulate Smad activation, but others might induce responses unrelated to transcription (Derynck and Zhang, 2003; Itoh et al., 2000).

Activation of ERK via TGFβ involves phosphorylation of tyrosine residues on both type I and type II receptors and/or on Shc. The phosphorylated tyrosines are capable of recruiting Grb/Sos which then activate Ras, Raf and their downstream MAPK cascades to finally activate ERK (Lee et al., 2007). Erk then regulates target gene transcription through its downstream transcription factors in conjunction with Smads to e.g. control epithelial to mesenchymal transition (EMT) (Derynck and Akhurst, 2007; Massague, 2008). In EMT TGFβ activated ERK is required for disassembly of cell adherence junctions and induction of cell motility (Davies et al., 2005; Zavadil et al., 2001). Besides, ERK can interfere with the Smad signaling cascade by regulating R-Smad activities through their phosphorylation (Kretzschmar et al., 1997; Kretzschmar et al., 1999; Matsuura et al., 2005). For instance,

Smad 2 is phosphorylated and thereby stabilized by ERK, which leads to enhanced Smad 2 transcriptional activity I mink lung cells (Funaba et al., 2002). Regarding apoptosis there are two biological links between receptor complexes and intracellular kinases involved. This is on the one hand the propoptotic adaptor protein Daxx, which leads to activation of JNK and induction of apoptosis in epithelial cells and hepatocytes (Perlman et al., 2001). On the other hand signals are transduced through the TGFβ activated kinase 1 (TAK1) leading to activation of p38. Whether there is activation of Smad or non-Smad pathways depends at least to some extend on oligomerization of the cell-surface receptors. For instance, preassembled type I and II receptors may activate Smads, whereas ligand induced receptor complexes may activate the TAK1-p38 cascade (Nohe et al., 2004). Overall, Smad-independent signals under the control of TGFβ provide a regulation of the signaling pathways and serve as nodes for crosstalk with other major signaling pathways such as tyrosine kinase, G-protein coupled or cytokine receptors. There is a high degree of cell-type specifity and many non-smad signaling proteins modulate the activity of the Smad pathway and thus the biological outcome (Moustakas and Heldin, 2005).

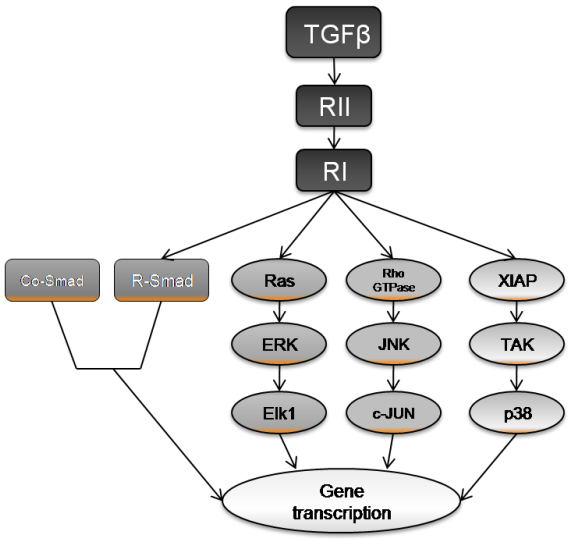

Fig. 5 TGFβ signaling cascade
TGFβ initiates the canonical Smad pathway as well as pathways including MAP Kinases like ERK, JNK and p38. (according to Itoh et al., 2000)

8. Regulation

In a complex system like a cell or a whole tissue, signals need to be finely tuned to ensure an appropriate response. This counts especially for a large group of proteins like the TGFβ family, which exerts a multiplicity of functions.

Different thresholds of activin A exposure, for example, can lead to contrasting cell responses, and even adjacent cell types can react differently (Kojima et al., 1993). Furthermore, only low amounts of the ligand are required to generate cellular response and responsiveness upon relatively low degrees of receptor occupancy suggest a need for activin A limitation at the receptor level. On the other hand the ligand has to be available in great amounts to fulfill paracrine and endocrine functions at distant sites, thus making a balance of the simultaneous need for both limiting and excess amounts of activin A essential (Phillips, 2000). This is achieved through the agency of cofactors, coactivator and corepressors and several other regulatory mechanisms at diverse biological levels. Thus the mix of factors present in a given cell at the time of TGFβ family ligand stimulation defines the final response.

8.1 Transcriptional Regulation

Regulation starts out on the transcriptional level, where inhibin and activin subunit expression is precisely controlled by cis-acting elements. However, the underlying mechanisms are incompletely explored. Only analyses of the inhibin and the activin βA promoter are known so far, revealing **TRE- and CRE-like transcription factor sites**, essential for transcriptional activation via TPA and cAMP stimulation. Thus, these elements may play a role in basal inhibin and activin βA gene expression and possibly also for inducibility (Ardekani et al., 1998; Tanimoto et al., 1996).

Transcription Factors

As Smad MH1 domain association with DNA is not selective and the interaction is of low affinity, so called transcription factors are necessary to provide a tight target gene specificity (Attisano and Wrana, 2000; Massague and Wotton, 2000).

The DNA binding proteins **FAST-1** (Chen et al., 1996; Chen et al., 1997) and **OAZ** (Hata et al., 2000) were the first identified Smad cooperators. Also previously known transcription factors that function independently of Smads in other contexts contribute to regulation. They do so either in a positive way like **AP-1** (JUN, Fos) (Zhang et al., 1998), the above mentioned **SP1** family members (Rouille et al., 1995) or **AML** proteins (Pardali et al., 2000); or in a negative way like the protooncogene product Evi1 (Alliston et al., 2005).

Transcriptional co-activators/co-repressors

Once the Smad complex binds to specific DNA elements it may activate the transcription by remodeling the chromatin structure with the help of co-activators, or it alternatively recruits co-repressors.

The structurally related **p300 and CBP** coactivators were shown to interact in a ligand-dependent manner with R-Smads through their MH2 domains (Feng et al., 1998; Nishihara et al., 1998; Pearson et al., 1999; Pouponnot et al., 1998). They enhance transcription by bringing the Smads into close proximity with the basal transcription machinery, or through their intrinsic histone acetylase activity, which loosens chromatin structure. A Smad interacting corepressor is **TGIF**, a homeodomain protein that can bind histone deacetylases (HDACs) and recruits other repressors (Wotton et al., 1999), thus inhibiting the promoter to which the Smad-TGIF complex is targeted. Additionally TGIF plays a role in negative regulation via the Ras-MEK-MAPK pathway (Lo et al., 2001). The highly conserved proto-oncogenes **Ski and SnoN** were also found to act as transcriptional corepressors for Smads by antagonizing TGFβ signaling through direct interaction with Smad4 and the R-Smads (Liu et al., 2001). **SARA**, another important Smad-interacting protein has already been mentioned above. It facilitates the access of R-Smads to activated TGFβ receptors via its central FYVE domain that mediates membrane association (Tsukazaki et al., 1998).

8.2 Postranslational Regulation

Intracellular Proteins and Co-factors

Once activated, the TGFβ family receptors are negatively regulated by the **I-Smads**, Smad6 and Smad7, which bind to the activated receptor in competition with R-Smad (Kavsak et al., 2000; Suzuki et al., 2002). The interaction also leads to ubiquitination and degradation of the receptor with the help of the **E3 ubiquitin ligases** and Smad ubiquitination regulatory factors (**Smurfs**) (Ebisawa et al., 2001; Tajima et al., 2003). Activin A, for instance, was shown to upregulate Smad7 mRNA within 3 hours, subsequently blocking downstream activin effects, thus indicating a negative feedback loop (Ishisaki et al., 1998). Since only dimeric activins are biologically active, subunit expression defines the repertoire of actually formed **activins** and **inhibins**. Thus, formation of inhibin A (αβA), inhibin B (αβB) and inhibin C (αβC) and activin heterodimers has regulatory functions as well. As inhibins and activins have antagonistic effects, altered expression of the α subunit could change the biological outcome. Furthermore, activin C is reported to modulate the synthesis of activin A and activin B via the formation of activin AC/AB heterodimers (Kobayashi et al., 2002).

Extracellular binding Proteins

The excess of TGFβ ligands to their receptors is amongst others controlled by extracellular binding proteins, which bind the ligand and therefore disable their access to the receptor. They include the **LAP** as well as the small proteoglycan **decorin** and the circulating protein **α_2-macroglobulin** binding to free TGFβ, **follistatin** which binds to Activin and BMPs, and **Noggin, Chordin** and **DAN/Cerberus** which also bind to BMPs and additionally has been shown to block Activin A binding to its receptor complex (McFarlane et al., 1999).

Pseudoreceptors

Activin receptor expression itself is a mechanism modulating activin signaling. For example expression of ActRIIB is down regulated after partial hepatectomy in rat livers, thus disabling apoptotic signals and enhancing liver regeneration (Gold et al., 2005). Aside altered receptor expression, so called pseudoreceptors promote ligand access to their actual receptor, but also binding of factors inhibiting receptor activation.

The membrane anchored **proteoglycan betaglycan**, also known as TGFβ type III receptor, has long been known to solely promote TGFβ binding to type II receptor (Brown et al., 1999; Massague, 1998). It does not appear to bind to activin or BMPs, however it was shown to bind the inhibin α subunit with high affinity, and to facilitate its access to ActRII. Since this so formed ternary complex is not able to recruit Alk4 and coincidentally prevents access of activin to ActRII, activin signaling is functionally inhibited (Lewis et al., 2000). **Cripto** and other membrane associated members of the CFG-EGF family are required for ActRII/ActRIIB and Alk4 receptor assembly and generation of signals induced by Nodal, VG1 and GDF1 (Cheng et al., 2003; Schier and Shen, 2000). Unlike these TGFβ family ligands, however, activin signaling is blocked by Cripto via its ability to associate with ActRII and blocking recruitment of Alk4 (Gray et al., 2003). The pseudoreceptor **BAMBI** (bone morphogenic protein and activin membrane-bound inhibitor) is a transmembrane protein with an extracellular domain that shares sequence similarities with type I receptors, and a short cytoplasmatic region lacking kinase activity, prohibiting its phosphorylation. Bambi competes with the type I receptor for association with ActRII/ActRIIB, therefore inhibiting receptor activation (Onichtchouk et al., 1999).

9. Activins in liver biology, homeostasis and regeneration

The activin subfamily, like other TGFβ family members, is involved in a wide variety of physiological processes including cell proliferation, differentiation and homeostasis (Hully et al., 1994; Schwall et al., 1993; Yasuda et al., 1993). While InhβA and InhβB mRNA is widely expressed in many tissues (Tuuri et al 1994, Vejda et al. 2002, the InhβC and InhβE subunit interestingly are abundantly expressed in the liver (Vejda et al. 2002, Hashimoto et al. 2002, Loveland et al. 1996, Gold et al. 2004, Thomas et al. 1998, Mellor et al. 2000). Here, the activins main source are the hepatocytes, which are the parenchymal cells making up the major part of the liver representing approximately 80% of the hepatic cells. The remaining 20% liver mass is composed of non-parenchymal cells, which include Kupffer cells, endothelial cells and hepatic stellate cells (HSC). TGFβ is produced mainly in non-parenchymal cell, but also InhβA mRNA expression takes place within these cells under pathological conditions (De Bleser et al., 1997). Under physiological conditions hepatocytes rarely divide. Not until partial hepatectomy or diffuse liver injury reduces liver mass, remnant liver cells reenter the cell cycle thus initiating regenerative DNA synthesis (Taub, 2004). However, not only cell proliferation is necessary to maintain the function of a multicellular organ, also mechanisms to terminate growth and mechanisms of programmed cell death are crucial to ensure a homeostatic balance. TGFβ family members are known to be part of a major paracrine/autocrine growth regulatory system, though occupying different functions in this process.

Activin A for example has been shown to be an autocrine growth inhibitor and to tonically inhibit proliferation of hepatocytes (Yasuda et al., 1993). Like TGFβ it inhibits mitogen induced DNA synthesis and induces apoptosis in hepatocytes in vitro and in vivo as well as in hepatoma cell lines (Hully et al., 1994; Schwall et al., 1993; Yasuda et al., 1993). In Hep3b cells activin A dependent apoptosis was induced by downregulating BcL-xL (antiapoptotic) expression via Smad2/3 (Kanamaru et al., 2002), whereas Smad2/3 and the activin receptors are known to mediate apoptosis also in HepG2 (Chen et al., 2000). In activin A treated HepG2 cells an increased expression of the cyclin dependent kinase inhibitor p15INK4B was involved in inducing cell cycle arrest (Ho et al., 2004). Treatment of the serum-starved human hepatoma cell line HLF with activin A antisense oligonucleotides confirmed the growth inhibitory function of endogenous activin A, as it stimulated cell proliferation (Takabe et al., 1999). The propapoptotic function of activin A was further confirmed by immunostainings of rat apoptotic hepatocytes positive for activin βA (Gold et al., 2005). Additionally, inhibin alpha-deficiency in mice causes cell death of hepatocytes around the central vein, which may be due to a 10 fold higher serum level of activin A (Matzuk et al., 1994). On the other hand overexpression of dominant negative type II

receptors for activin A as well as TGFβ and therefore blockade of the corresponding downstream signaling led to stimulation of DNA synthesis (Ichikawa et al., 2001). Administration of the activin A antagonist follistatin in vivo by intraportal infusion or adenovirus-mediated overexpression caused liver mass and DNA content growth, by blocking the effect of activin A (Kogure et al., 2000). Notably, the remnant liver function and reorganization of liver architecture was better in control and activin treated rats (Endo et al., 2006). This may be due to differentiation inducing activity of activin A in hepatocytes, as well as its ability to stimulate non-parenchymal cells. For example, tubulogenesis of sinusoidal endothelial cells is augmented by activin A, and it stimulates collagen and fibronectin production in HSC, all supporting formation of extracellular matrix important during liver regeneration (Date et al., 2000; Endo et al., 2004; Wada et al., 2004a). After partial hepatectomy, the expression of the InhβA subunit drops abruptly to less than 50% of control values, and increased again to three times above control after 168h when liver mass was fully restored. The expression of ActRIIB, the activin A high affinity receptor, resembles that of βA (Gold et al., 2005; Takabe et al., 1999; Yasuda et al., 1993). Takamura et al. and Dater et al. confirmed the synchronical expression of activin A and its receptor, though report the recovery of expression already between 48h and 72h after PHX (Date et al., 2000 (Takamura et al., 2005). However, Mashima et al. report the effects of activin A to be very much dependent on the intracellular activin-follistatin system, as well as on cell density at which test are carried out (Mashima et al., 1995). Furthermore, there are reports that credit activin A with mitogenic functions in some cellular environments. Hedger et al. for example found FR3T3 fibroblast to proliferate upon activin A treatment (Hedger et al., 1989; Kojima et al., 1993).

Much less is now about the roles of the other activin subfamily members in liver biology. **Activin B**, unlike activin A and interestingly also unlike the heterodimer AB, did not inhibit DNA synthesis in primary cultured rat hepatocytes (Niimi et al., 2002). Nevertheless, rat FaO and human Hep3b cells infected with ALK7, which is supposed to be the preferred type I receptor of activin B, undergo apoptosis. Furthermore, Smad2/3, the MAP Kinases JNK and p38 and Caspase 3/9 were activated, indicating a cross-talk with the cellular stress death pathway (Kim et al., 2004a).

Though InhβC and InhβE mRNA are mainly expressed in the liver, knock-out mice lacking either one or both subunits did not show any abnormalities in liver size and function (Fang et al., 1997; Hashimoto et al., 2002; Lau et al., 2000). However, conflicting data were gained regarding **activin C**s effect on cell growth, DNA synthesis and apoptosis, depending on the cellular system worked with. An immediate down regulation of βC mRNA following PHX, supposed a similar inhibitory effect on cell proliferation for activin C as for activin A (Esquela

et al., 1997; Zhang et al., 1997). Chabicovsky et al. were the first to report an inhibiting effect of activin C, as well as activin E, on DNA synthesis in vivo (Chabicovsky et al., 2003). Accordingly, transfection of the human hepatoma cell lines HepG2 and Heb3b and the rat hepatoma cell line H4IIEC3 with activin βC and βE caused reduced cell growth, enhanced apoptosis and elevated levels of active Caspases (Vejda et al., 2003). All these results demonstrate activin C to inhibit proliferation and induce apoptosis just like activin A and TGFβ. In direct contrast, Wada et al. found exogenous activin C to stimulate AML12 hepatocyte and primary rat hepatocytes cell number and supported DNA synthesis (Wada et al., 2004b). Consistent with that activin C immunoreactivity in regenerating liver was found mainly in mitotic hepatocytes (Gold et al., 2005). In addition adenovirus-mediated expression of mouse InhβC augmented liver regeneration after PHX in rats (Wada et al., 2005a). However, the mechanism promoting cell proliferation following activin C administration may be due to reduced formation of activin A homodimers.

Activin E, like activin A, is reported to have growth limiting functions. It has been shown to negatively regulate cell growth in human hepatoma cell lines and murine hepatocyte cell lines (Vejda et al., 2003; Wada et al., 2005b). Furthermore, its transient overexpression by non-viral gene transfer inhibited regenerative DNA synthesis in mice (Chabicovsky et al., 2003). After partial hepatectomy, InhβE mRNA expression was increased rapidly and decreased to near basal levels after 48 hours (Takamura et al., 2005).

10. Activins in liver diseases and cancer

Activins, their receptors and antagonistic proteins are part of a well balanced system, determining the biological effect of this subfamily of proteins. Deregulation of it contributes to pathologic dysfunctions like hepatic inflammation, fibrogenesis and tumorigenesis (Rodgarkia-Dara et al., 2006).

Activin A has been related to pro- as well as anti-inflammatory effects. During systemic inflammation it gets significantly elevated (Jones et al., 2000), and is even released earlier than tumor necrosis factor alpha and IL-6 (Jones et al., 2004). Both of them are under the earliest cytokines released in inflammation. In the hepatoma cell line HepG2 activin A influences inflammation by inhibiting the IL6 induced APP synthesis (Werner and Alzheimer, 2006). A similar action has been found for activin C. Proteome analysis of primary rat hepatocytes treated with inducers of APP (acute phase proteins) followed by human recombinant activin C treatment revealed alterations in synthesis of numerous proteins. These included decreases in secreted APP T-kininogen, fibrinogen β- and γ-chain and fetuin

A while synthesis of transthyretin, albumin and apolipoprotein A-1 was increased (Erlach et al, in preparation).

Liver inflammation is closely linked to fibrosis, thus several reports document an involvement of activins in this disease too. Activated HSC in fibrotic and cirrhotic rat livers were found to express elevated levels of activin A (De Bleser et al., 1997; Patella et al., 2006). Furthermore, expression of InhβA mRNA was increased in rat liver fibrosis and cirrhosis induced by injection of dimethylnitrosamine or porcine serum (Sugiyama et al., 1998). Elevated levels of circulating activin A were found in patients suffering from chronic viral hepatitis or alcohol induced liver cirrhosis (Elsammak et al., 2006; Patella et al., 2001; Pirisi et al., 2000; Yuen et al., 2002). Finally, activin A contributes to fibrosis by induction of actin, fibronectin and type I collagen synthesis in HSCs (Date et al., 2000; Wada et al., 2004a). While hepatic InhβE expression was elevated after treatment with LPS, InhβA, InhβC and InhβE expression was upregulated during CCl_4 induced fibrosis in rat livers (De Bleser et al., 1997; Gold et al., 2003; Huang et al., 2001).

Chronic tissue inflammation leading to fibrosis and cirrhosis usually are the precursors for hepatocellular carcinoma, which are the leading causes of cancer deaths in many parts of the world (Caldwell and Park, 2009; Drucker et al., 2006; Grasl-Kraupp et al., 2000; Herzer et al., 2007; Seitz and Stickel, 2006). There is more and more evidence that activins are involved in the process of liver carcinogenesis. As activin A exerts growth inhibitory and proapoptotic effects on most epithelial cell types including hepatocytes, escape from its control may be necessary for tumor development. In chemically induced tumor tissues InhβA mRNA expression was reduced in 5 out of 11 HCC individual samples (Grusch et al., 2006). On the other hand, increased levels of circulating activin A were found in HCC patients (Elsammak et al., 2006; Patella et al., 2001; Pirisi et al., 2000; Yuen et al., 2002). Furthermore, activin A has been associated to neoangiogenesis because it stimulates VEGF expression in human hepatoma cells (Wagner et al., 2004). For activin B, which role in the liver is not well characterized, no data regarding expression changes during hepatocarcinogenesis have been reported yet. No data regarding the liver specific activin C are known either. InhβE subunit expression, like InhβA, was found to be reduced in human HCC samples (Grusch et al., 2006).

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II. Aims of the thesis

Research for a long time focused on activin A, neglecting the highly liver specific activin C. The major aim of this thesis was to investigate mRNA expression of the TGFβ family members in diverse human tissues and to investigate signaling mechanisms of activin C and its involvement in different biological functions.

1. In the first manuscript, we determined the mRNA expression pattern of the inhibin gene family to obtain novel insights into possible functions of activin and inhibins in human hepatocellular carcinogenesis. α, βA, βB, βC and βE subunit mRNA levels in normal liver samples, hepatocellular carcinoma and adjacent fibrotic/cirrhotic tissue specimens were assessed by quantitative real-time PCR analysis and compared to reference hepatoma cell lines. Additionally, we aimed to determine different human tissues to obtain a hint for the comparable expression distribution between rodents and humans.

2. Although the signaling cascade for activin A is well investigated, receptors and downstream signaling for activins containing βC or βE subunits are hardly elucidated so far. The aim of the second study was to elucidate signaling transduction for activin C, thereby not only focusing on Smad but also on Smad independent mechanisms. Additionally, we were interested in the consequences of differing signaling modes on viability and apoptosis.

3. The objective of the third part was to shed light on some aspects of TGFβ family members' fields of action. They are involved in many biological mechanisms, like embryonic development, inflammation, cell proliferation, apoptosis and thus metastasis and cancerogenisity. It is also an approach on studying activin C, for which, in contrast to activin A, hardly anything is known so far about its impact on physiological mechanisms.

III. Results and discussions

1. Manuscript I

Expression of the Inhibin/Activin Gene Family in Human Hepatocellular Carcinoma

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Abstract

Activin and inhibin, members of the inhibin gene family, belong to the TGFβ superfamily, which controls cell proliferation and differentiation in many organs. Activin A is a potent anti-proliferative agent in hepatocytes and may affect tumour formation. The role of the other inhibin subunits, α, βB, βC and βE, in human liver and hepatocellular carcinomas (HCC) is largely unknown. In this study we examined changes in mRNA expression of the complete inhibin gene family in normal, fibrotic/cirrhotic and malignant liver tissue. For this purpose quantitative mRNA expression of inhibin genes was investigated in samples of HCC and tumour surrounding tissue obtained from sixteen patients after surgery. Samples from ten disease-free donors were used as reference. Additionally twelve different organs and rodent livers were analysed. Expression of inhibin subunits was determined by quantitative Real-Time polymerase chain reaction. Normalization procedure was performed using geNorm algorithm. All four β subunits were expressed in normal and patient samples at similar levels. βC subunit expression was unaltered in HCC patients compared to disease-free liver samples. All four β subunits were lower in adjecent tissue than in disease-free or HCC samples, whereas expression of α subunit increased steadily from normal to malignant samples. The inhibin family expression level differs in rodent liver. Immunostaining for inhibin α, barely detectable in normal liver, was strongly increased in tumor samples. The described upregulation of inhibin α and βB subunit may serve to antagonize the antiproliferative effect of βA.

Introduction

Liver cancer is among the leading causes of cancer deaths worldwide. It is characterized by deregulated proliferation and apoptosis of hepatocytes and usually develops on the basis of chronic tissue inflammation leading to fibrosis and cirrhosis (Drucker et al., 2006; Grasl-Kraupp et al., 2000; Herzer et al., 2007; Seitz and Stickel, 2006). The underlying molecular mechanisms are barely understood (Laurent-Puig and Zucman-Rossi, 2006; Macheiner et al., 2006; Strand et al., 1996) whereas their elucidation is critical for the development of improved therapies for liver cancer (El-Serag and Rudolph, 2007; Llovet et al., 2003; Schulte-Hermann et al., 1997).

Activin and inhibin proteins are members of the TGFβ superfamily, which control cell proliferation, apoptosis, inflammation and differentiation in many cell types and organs including the liver (Deli et al., 2008; Rodgarkia-Dara et al., 2006; Schulte-Hermann et al., 1997). The mammalian inhibin gene family includes one α and four β subunits, namely INHBA, INHBB, INHBC and INHBE. Inhibin proteins are dimeric glycoproteins consisting of an α subunit and one of the β subunits, whereas activin proteins are composed of two β subunits. Earlier expression studies at the mRNA level revealed that the α subunit INHA is most prominently expressed in ovary, testis, adrenal and pituitary gland but absent in the liver (Meunier et al., 1988; Tuuri et al., 1994). Expression of INHBA mRNA is high and widely distributed in many organs including the liver. Likewise, INHBB mRNA has been detected in many organs too. However, in rodent liver it was shown only at low or undetectable levels or to be transiently induced upon diverse treatments therein (De Bleser et al., 1997; Jones et al., 2007; Kobayashi et al., 2002; Kobayashi et al., 2000; Vejda et al., 2002a). In contrast, INHBC and INHBE mRNA expression levels are highest in the liver, much lower in testis, adrenal and pituitary gland and almost undetectable in other organs of rodents (Fang et al., 1997; Gold et al., 2004; Vejda et al., 2002a).

The resulting activin proteins may have important functions in the liver, but with the exception of activin A these functions remain to be clarified. Activin A is considered as a key inhibitor of liver growth (Xu et al., 1995; Yasuda et al., 1993; Zauberman et al., 1997), whereas the so-called liver specific activins, activin C and activin E, were reported to either promote (Wada et al., 2004b; Wada et al., 2005a) or inhibit hepatocyte cell growth (Chabicovsky et al., 2003; Vejda et al., 2003). After partial hepatectomy in mice nulled for INHBC or INHBE or for both genes no alterations in liver regeneration occured (Lau et al., 2000). Studies on expression of INHBC and INHBE mRNA during liver regeneration after partial hepatectomy (Esquela et al., 1997; Gold et al., 2005; Kogure et al., 1998; Lau et al., 2000; Takamura et al., 2005; Wada et al., 2005a; Zhang et al., 1997) or chemical injury (Gold et al., 2003; Grusch et al., 2006; Kobayashi et al., 2002) in rodents produced conflicting data on possible biological

functions as reviewed in (Grusch et al., 2007). Most of the information available were gathered in rodent liver and rarely confirmed in human (Tuuri et al., 1994).

Recently we found that INHBA and INHBE were downregulated in chemically induced hepatocarcinogenesis in rats (Grusch et al., 2006). In patients with cirrhotic and hepatocellular carcinoma (HCC) however, elevated levels of serum activinA were observed and were suggested as biomarker of liver disease (Pirisi et al., 2000; Yuen et al., 2002). Likewise, in several other malignancies deregulation of inhibin and activin family members on the mRNA and protein level is a common event, including tumors of endometrial (Worbs et al., 2007), adrenocortical (Hofland et al., 2006; Hofland et al., 2007; Salmenkivi et al., 2001) and gonadal stromal origin (Ciris et al., 2004; Fine and Li, 2003; Fuller et al., 1999).

Consequently, in the present study we performed a systematic analysis of the mRNA expression pattern of the inhibin gene family in human HCC. For comparison reasons, these levels were also analysed in different human tissues and in mouse and rat liver. For comprehensive quantitative Real-Time PCR analysis mRNA levels were assessed in samples from normal liver, carcinoma and adjacent fibrotic/cirrhotic tissue specimens and normalized by GeNorm as described in Frost et al. (Frost et al., 2010). This provides a distinct molecular expression portrait of the inhibin gene family in disease-free livers and HCC patients, in other organs and rodent liver. To our surprise, the mRNA of the α subunit, barely detectable in disease-free and tumor adjacent liver samples, was dramatically elevated in the tumor samples. INHBB mRNA, almost undetecable in rat liver, was highly expressed in disease-free human liver and further upregulated in HCC. To our knowledge this is the first analysis of the complete inhibin gene family in human healthy liver and hepatocellular carcinoma.

Material and methods

Human liver samples, tissue mRNAs and rodent liver

Tumour (T) and tumour adjacent liver (non-tumour, NT) tissue samples (n=16) from HCC patients plus five disease-free, normal (N) liver samples from donors were obtained from the General Hospital, Vienna as part of the Austrian 'Gen-Au Programme' (study no. GZ 200.058/6-VI/2/2002), described in detail in (Grusch et al., 2006). As this study was actually concepted for genomic DNA and histological analysis, and due to restricted amount of tissue, RNA was only prepared from a subset. Written informed consent was obtained from each patient. Additionally, five samples of RNA from normal liver were purchased from Stratagene, Clontech and BioCat. Thereby, the total number of normal liver samples was 10.

Histological analysis of H&E-stained sections showed that all cancer samples contained at least 80% tumor cells. Tumor stage and grade were determined according to AJCC/UICC standards and Edmondson-Steiner (Edmondson and Steiner, 1954), respectively. Six of the 16 tumours were classified as stage I, seven tumours as stage II, one tumor as stage III, and two tumours as stage IV. The histological grading was two HCC-1, 13 HCC-2, and one HCC-3. The surrounding tissue was cirrhotic in seven cases and fibrotic in nine cases. Further patient information are listed in Table 1.

Human tissue RNAs from further 12 organs were purchased from BioCat and Stratagene. Liver samples from C57BL/6 mice and Wistar rats were obtained as described previously (Grusch et al., 2006) .

RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Liver tissue samples were prepared using a homogenizer (Bertin Technologies). High quality of RNA samples and reproducibility of assays were carefully controlled on GenQuant (Pharmacia). All samples of RNA were protein-free as indicated by a ratio of 1.8 – 2.1 of absorbance at 260/280nm. The mean error of repeated RNA concentration measurements (n=3) of all samples (n=42) was 8.0%. The integrity of all RNA samples was confirmed electrophoretically on 1.5% agarose gel.

Reverse Transcription and Quantitative Real-Time PCR

Reverse transcription was performed up to three times following photometrical determination of RNA concentration. The mean error of those repeated RNA measurements was 8.0%. Two micrograms of total RNA were reverse transcribed with MMLV RevertAid (Fermentas) resulting in 100µL cDNA solution. Two microliters cDNA were used as a template for each PCR. Real-Time analysis was performed with TaqMan® Universal PCR Master Mix (Applied

Biosystems) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) running the following protocol: initial 50°C 2min and 95°C 10min, and 40 repeats of 95°C 15 sec, 60°C 1 min. Taqman® assays for human inhibin α (INHA; Hs00171410_m1), βA (INHBA; Hs00170103_m1), βB (INHBB; Hs00173582_m1), βC (INHBC; Hs00173745_m1) and βE (INHBE; Hs00368884_g1) were obtained from Applied Biosystems. For geNorm based normalization calculation β-actin (ACTB; Hs99999903_m1), β2-microglobulin (B2M; Hs99999907_m1), glycerinaldehyde-3-phosphate dehydrogenase (GAPDH; Hs02758991_g1), hypoxanthine phosphoribosyl-transferase1 (HPRT; Hs99999909_m1) and TATA box binding protein (TBP; Hs00427620_m1) were used. All samples were analysed in duplicate and in at least two independent Real Time-PCR runs. The intra-assay mean error was 0.16% and the inter-assay mean error 1.24%.

Calculation of expression data

Original (raw) cycle threshold values C_t were transformed by the following procedures. For each gene, the median C_t value of data from normal liver samples (n=10) was used as calibrator. Each single sample C_t value was logarithmically transformed, calculated by the formula $2^{-(\text{calibrator-sample})}$, and expressed as fold change. For normalization procedure, samples were transformed as described in (Vandesompele et al., 2002). Since expression of standard refernce genes in normal and malignant liver showed large variations we determined the most stable one using geNorm applet. We used the hence determined ones, for disease-free samples B2M, ACTB and HPRT, for HCC samples GAPDH, HPRT and TBP (Frost et al., 2010). Additionally, HCC samples where normalized in two different ways by calculating geNorm normalization factors NF for all NT and T tissue samples jointly or seperately as individual subgroups. After normalization calculation, by dividing transformed data with the respective NFs, the median of normalized data from disease-free liver samples was used as calibrator and set to 1. Raw C_t values, where total RNA input was assumed as normalization basis, and the values determined by the two different normalization approaches were listed in Table 2. For species and tissue comparisons raw C_t values were used. The lowest expressed sample was used as calibrator, and set to 1 upon logarithmical transformation. Results from all other samples were expressed as fold change.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 4.0 for Windows (www.graphpad.com). Data of all three groups (N, NT and T) were analysed by Kruskal-Wallis test. Significance of differences between medians of non-paired groups (N and NT, N and T) were checked using the non-parametric Mann-Whitney U-tests. Results from paired NT and T samples were analysed by the non-parametric Wilcoxon matched pairs test, since

gene expression levels not always showed normal distribution. Correlation between gene expression levels and ratios was performed by Spearman ranked test. All tests were performed as two-tailed and statistical significance was assumed at $p< 0.05$

Immunohistochemistry

Histological staining was performed as previously described (Grusch et al., 2006). In brief, tissue samples were fixed in 4% buffered formalin embedded in paraffin, 2µm sections were deparaffinated, and antigen-retrieval was done by heating in 0.01 M citrate buffer. Sections were incubated in 0.1% BSA/PBS with primary antibodies against inhibin α (Neomarkers) and inhibin βB subunit (R&D Systems) diluted 1:100 overnight at 4°C. Then, sections were washed in PBS with 0.5% Tween 20 and incubated with HRP-coupled secondary anti-mouse antibody (Dako) diluted 1:200. DAB (Dako) was used as chromogen to detect peroxidase activity. Sections were counterstained with hemalaun and mounted in Dako mounting medium (Merck). As positive control for inhibin α and inhibin βB subunit protein expression histological sections of ovarian tumors (Biogenex) were used.

Results

Inhibin gene family expression in disease-free liver and HCC - assessing C_t values

We have previously studied INHBA and INHBE expression changes in two samples of human disease-free liver and 11 samples of HCC, wherein both genes were described as downregulated in tumor samples (Grusch et al., 2006). We now extended our analysis to all inhibin family members and to adjacent, fibrotic or cirrhotic HCC patient tissue. Furthermore, we increased the sample numbers by additional five HCC (n=16) and eight disease-free liver (n=10) specimens. As B2M was used as reference genes in the previous work, we also analysed its expression in this study (Fig. 1).

Inhibin α subunit mRNA was reported to be undetectable in rodent liver (De Bleser et al., 1997) and to our knockwlegde until now no real time expression data are available from human liver. Inhibin α mRNA was detectable in 7/10 disease-free liver samples with C_t values between 33.5 and 36.5, 15/16 adjacent tissues with C_t values between 30.5 to 39.5 and in all HCC tumor samples with C_t values between 24.5 to 36. Its expression was significantly upregulated from disease-free (N), to adjacent (NT) and tumor (T) tissue. Expression of the four β subunits, INHBA, INHBB, INHBC and INHBE was detectable in all liver samples with C_t values from 20.5 to 35.5. Based on C_t values, the median expression of INHBA, INHBB and INHBE was found upregulated from NT to T samples, whereas INHBC expression level was unaltered. These preliminary data are in contrast to previous work (Grusch et al., 2006). When analysing the expression of the formerly used reference gene B2M a strong upregulation from disease-free to adjacent and tumor sample was detected. If using the expression level of B2M as single reference it falsifies the results obtained for the genes of interest. Therefore we choose additionally to raw C_t data the use of multiple reference gene expression and geNorm calculation for further in-depth analysis in human liver and HCC samples (Frost et al., 2010).

Normalization and evaluation of Inhibin gene family expression data

For a comprehensive gene expression analysis normalization strategies are recommended. One possibility is to set the amount of high quality input RNA as reference (Tricarico et al., 2002) and directly use C_t values or data derived by logarithmical transformation calculation, refered to as ‘without normalization’. As another normalization method we applied a combination of multiple reference genes determined by geNorm (Vandesompele et al., 2002) as described in detail in a previous study (Frost et al., 2010). Briefly, we used two different sets of normalization factors determined by either assuming HCC samples NT and T jointly as one group, referred to as ‘NF jointly’ or separated them into the subgroups NT and T, referred to as ‘NF separately’ (Table 2). This range of data for each gene appeared as

reliable approach to evaluate expression changes between disease-free, adjacent and tumour tissue particularly with regard to unstable expressed reference genes.

In the present study the median expression of disease-free normal liver samples was used as calibrator and set as 1 and the expression levels in NT and T samples were calculated as fold increase (Table 2). The range of median expression level for INHA in tumour samples was between 5.2 and 8.7, which was significantly elevated in relation to disease-free liver and NT samples. All four inhibin β subunits tended to be downregulated without statistical significance in adjacent tissue (NT). No statistically significant expression changes were found in HCC samples (T) compared to normal disease-free liver. INHBA and INHBB expression was significantly upregulated by 1.7- to 4.2-fold and 3.5- 5.4 fold, respectively, in tumor samples in comparison to adjacent tissue. For INHBE expression an increase in tumour samples compared to their paired adjacent samples was significant only in two of the three normalization methods. Also a decrease without statistical significance of median INHBE expression in tumor samples in comparison to normal liver was determined by two of the three normalization methods. This supports our previous finding, that INHBE may be downregulated in hepatocellular carcinoma (Grusch et al., 2006). The slight INHBC median expression changes ranged between 0.7 and 1.4 and did not reach significancy. The expression of the INHBC was therefore considered as unaltered in this hepatocellular carcinoma patient population.

Assessing inhibin family expression regulation for individual patients

In microarray expression studies the use of a threshold is common, because it often works reasonably well for ranking results (Allison et al., 2006). From this point of view we evaluated our generated data, respective the ratio of T/NT of log transformed raw C_t values. Ratios between a threshold of 0.5 and 2 are considered as unaltered, lower than 0.5 as downregulated and higher than 2 as upregulated (Fig.2A). In 11/16 patients none of the five inhibin family members was downregulated and 2/16 none of them was found upregulated.

INHBA and B2M were in 11 and 10 patients upregulated and the only two not found to downregulated in this collective of HCC patients. INHA and INHBB were upregulated in 8 and 9 and downregulatred in 2 patients, respectively. Also with this kind of data interpretation the most constantly expressed inhibin family member was INHBC (Table 3). As INHBA is well known as regulator of hepatocyte growth (Grusch et al., 2007) and also the resulting activin A protein is considered as clinical marker for HCC (Pirisi et al., 2000; Yuen et al., 2002), our data fit well into this established pool of knowledge.

In view of the unexpected high expression and upregulation of INHA and INHBB in a subset of HCC patients (Fig.2B) we took a closer look at individual patients and possible gene expression correlations. In one patient both genes were found downregulated from NT to T.

No correlation between those both genes could be determined. A strong expression correlation between INHBB and INHBC was calculated for non-tumor ($r=0.897$, $p= 3x10^{-6}$) and tumor ($r=0.924$, $p= 6x10^{-7}$) samples and also for INHA and INHBA in non-tumor ($r=0.782$, $p= 0.0006$) samples only by Spearman ranked test. Gene expression level or ratio T/NT did not correlate with other clinical parameters as gender, age, viral and fibrotic/cirrhotic status.

Inhibin α and βB protein expression in HCC

To confirm expression of INHA and INHBB on protein level, we searched for the presence of the respective proteins in some tumour samples. For the detection of the inhibin α protein subunit we choose histological sections of the patient with the highest mRNA level in the tumour area, as we expect to work at the detection limit in liver tissue. For inhibin βB protein subunit detection we chosse the patient with the highest tumor/non-tumor ratio and with a basal level for NT section comparable to normal livers at our disposals (Fig.2B, arrows).

We found strong cytoplasmic immunostaining for inhibin α in the carcinoma cells (Fig.3b), whereas protein levels were below detection limits in the corresponding adjacent liver tissue (Fig.3a). βB subunit immunostaining in adjacent and tumor tissue was strong in cells from parenchymal origin, again corresponding to mRNA expression levels (Fig.4a and b). In both cases human ovarian tumor sections were used as positive control (Fig.3c and Fig.4c). Omission of primary antibodies resulted in complete loss of staining (Fig.3d and Fig.4d).

As the antibodies detect the respective subunits only, no conclusion about the actual composition of the dimeric inhibin or activin protein can be drawn.

Inhibin family gene expression differs in human and rodent liver

Unlike in rat liver (Vejda et al., 2002a), the INHBB subunit was found as highly expressed as the so-called liver specific subunits INHBC and INHBE in human liver samples. Therefore we analysed inhibin family expression in mouse and rat liver. The lowest expressed gene, rat INHBB, was used as calibrator and set to 1, the expression levels of the other genes were depicted as fold increase in logarithmic scale (Fig.5). To exclude PCR based C_t value differences between genes and species, distinct plasmid dilution series were performed and revealed detectablilty at similar Ct levels (data not shown). In rodent liver INHBC and INHBE are predominantly expressed as expected. In human liver INHBA and INHBB were also highly expressed, with predominance of INHBB and INHBE followed by INHBC and INHBA. In mouse, both INHBA and INHBB were expressed at similar rather low levels. In rat liver INHBA expression was almost as high as in human while INHBB expression was very low, detectable at C_t 37.5. INHA expression, overall at a low level, was about 10-fold higher in rodent than in human liver (Fig.5). Taken together, there are profound interspecies variations

in basal hepatic expression of inhibin genes, which should be taken into consideration when extrapolating results from rodents to human.

Inhibin gene family expression in human tissues

In order to put the expression data in human liver into perspective, we analysed inhibin family member expression in 12 additional human organs (Fig.6). For comparison expression levels of disease-free liver and HCC are included in the diagrams. The lowest expressing tissue was used for calibration and set to 1, the expression levels in all other tissues were depicted as fold increase in logarithmic scale. As reported previously, α subunit INHA expression was highest in testis (Meunier et al., 1988; Tuuri et al., 1994), exceeding levels in pancreas about 10-fold, in heart about 100-fold, in HCC about 250-fold and in disease-free liver about 2500-fold (Fig.6). All four β subunits were highly expressed in the liver. The INHBA and INHBB subunits were detected in almost every tissue sample with a remarkable similar overall pattern, and highest expression in liver. INHBC and INHBE expression were at least 100-fold higher in liver than in any other organ investigated, but nevertheless detected in 11/13 and 9/13 tissues. Thus, INHBC and INHBE are predominantly, but not exclusivly expressed in the liver as assumed previously.

Table 1. Characteristics of normal and HCC-patient tissue samples

	Normal	HCC-patients
Number of samples	10	16
Sex-female	4	6
male	6	10
Age at operation (mean±SD, range)	50.5±6.6 45-64	63.4±12.4 43-78
HBV infected	0/10	0/16 (0%)
HCV infected	0/10	5/16 (31%)

Table 2. Changes in expression of Inhibin genes in HCC samples calculated with different normalization methods

	without normalization		NF separately		NF jointly	
	NT	T	NT	T	NT	T
INHA	7.679±2.478 3.942	179.9±118.2 8.695	4.172±1.821 1.769	98.15±56.58 6.680	4.744±2.070 2.012	139.5±94.25 5.173
	Wmp*, MW-NT*, MW-T**		Wmp**, MW-T*		Wmp**, MW-T*	
INHBA	0.6122±0.135 0.563	1.940±0.460 0.969	0.6766±0.173 0.551	6.271±3.692 2.323	0.7693±0.196 0.627	3.912±1.449 1.797
	Wmp*		Wmp**		Wmp**	
INHBB	3.986±1.741 1.226	11.91±4.034 4.320	2.474±1.030 0.635	30.22±18.99 3.484	2.813±1.171 0.722	16.30±7.624 4.304
	Wmp*		Wmp**		Wmp**	
INHBC	1.295±0.509 0.734	1.652±0.524 0.980	1.111±0.375 0.711	3.390±1.451 1.258	1.263±0.426 0.809	2.188±0.640 1.448
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INHBE	1.193±0.329 0.943	3.118±1.567 1.657	0.795±0.325 0.401	3.098±1.149 0.758	0.904±0.369 0.456	2.040±0.722 0.650
	Wmp*		Wmp**		---	

Values are given relative to the median mRNA expression value in normal liver tissue. Data are indicated as mean ± SEM and the median values.
Wmp...Wilcoxon matched pair test; MW-NT...Mann-Whitney U test comparing N and NT samples; MW-T...Mann-Whitney U test comparing N and T samples; * p-value<0.05; ** p-value< 0.01

Table 3. Evaluation of Inhibin family member and B2M regulation in HCC patients

	up >2-fold	equal 0.5- to 2-fold	down <0.5-fold
INHA	8	6	2
INHBA	11	5	0
INHBB	9	5	2
INHBC	3	10	3
INHBE	7	8	1
B2M	6	10	0

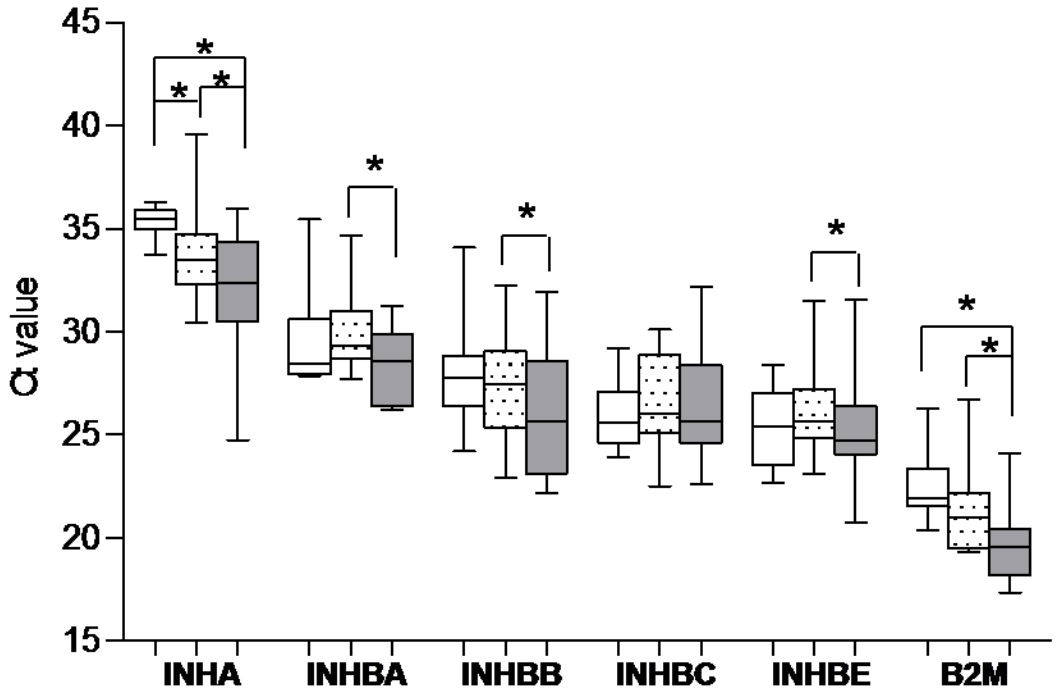

Fig.1 mRNA expression levels of the inhibin gene family and B2M in paired non-tumour adjacent (NT) and tumour (T) tissues and disease-free liver (N) tissue

Values are given as Real -Time PCR cycle threshold numbers (C_t value). Boxes (blank: N, dotted: NT; grey: T) represent the lower and upper quartiles with medians; whiskers illustrate the 10 to 90 percentiles of the samples. Expression differences in paired HCC patient samples were analysed by Wilcoxon matched pairs test, respectively Mann-Whitney U test for comparison of disease-free liver samples and NT and T samples. Significant changes ($p<0.05$) are marked with brackets and asterisk.

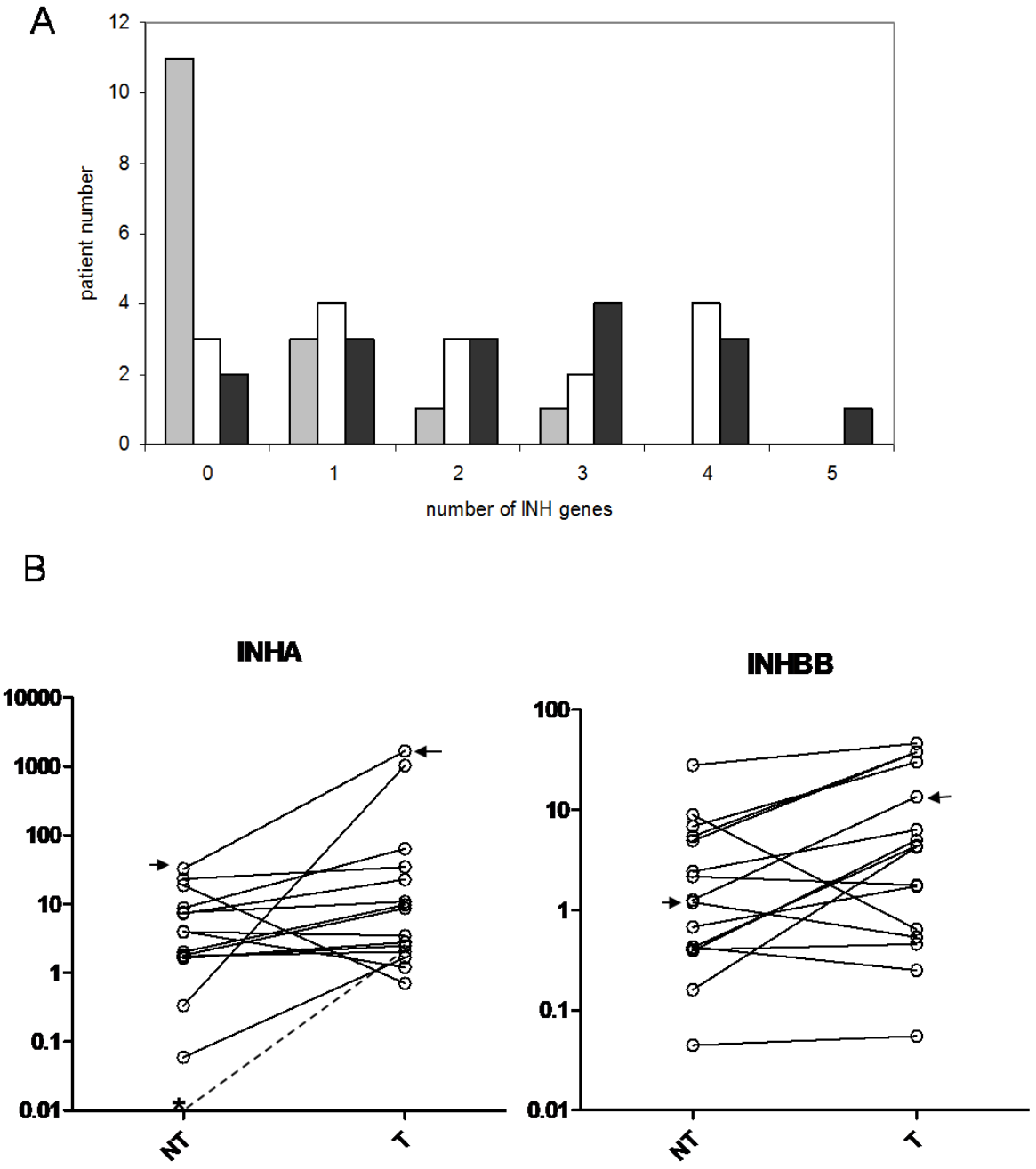

Fig.2 mRNA expression levels and changes of INHA and INHBB in individual patients
(A) The numbers of patients with different quantities of down (gray bars), up (black bars) or non-regulated, equally (white bars) expressed inhibin family genes are depicted. A treshold between 0.5 and 2 was considered as non-regulated. (B) Values are given as logarithmically transformed C_t values, where the median expression of disease-free liver samples is set as 1. Paired samples of NT and T are connected with a line. The asterisk marks one undetectable NT sample and the dotted line connects it with the paired T sample. The arrows mark chosen patients for immunohistochemistry.

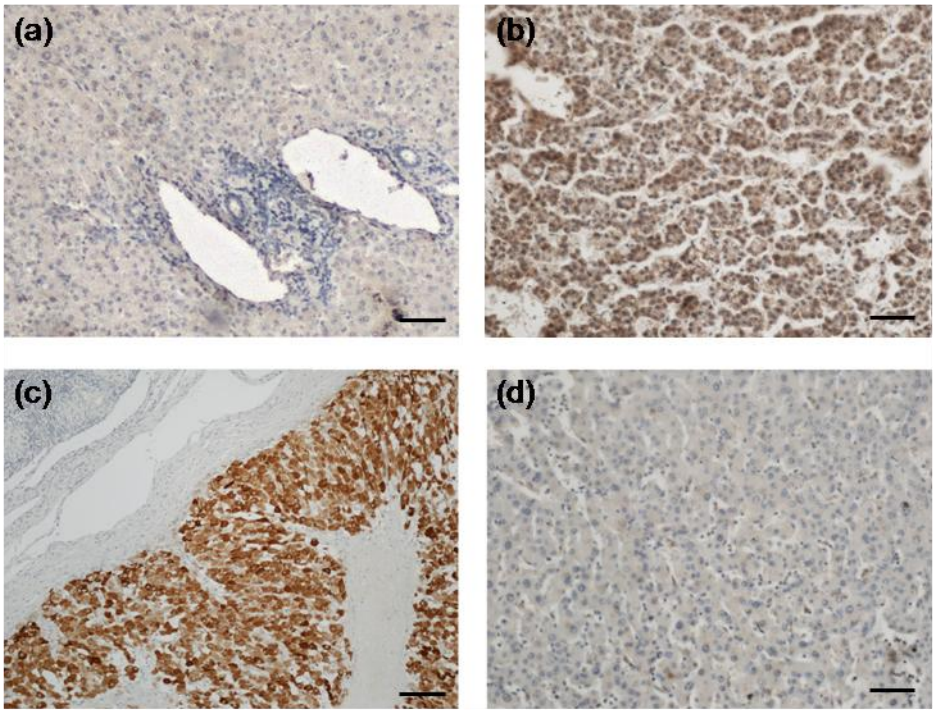

Fig.3 Immunohistochemistry of the inhibin α subunit in HCC patient's liver sections
No inhibin α is detectable in the adjacent non-tumour area (a) and positive staining is obtained in the tumour area (b). A tissue section of ovarian carcinoma was used as positive control (c). The primary antibody was omitted on this HCC tumor sectionb (d). Bars represent 250 µm.

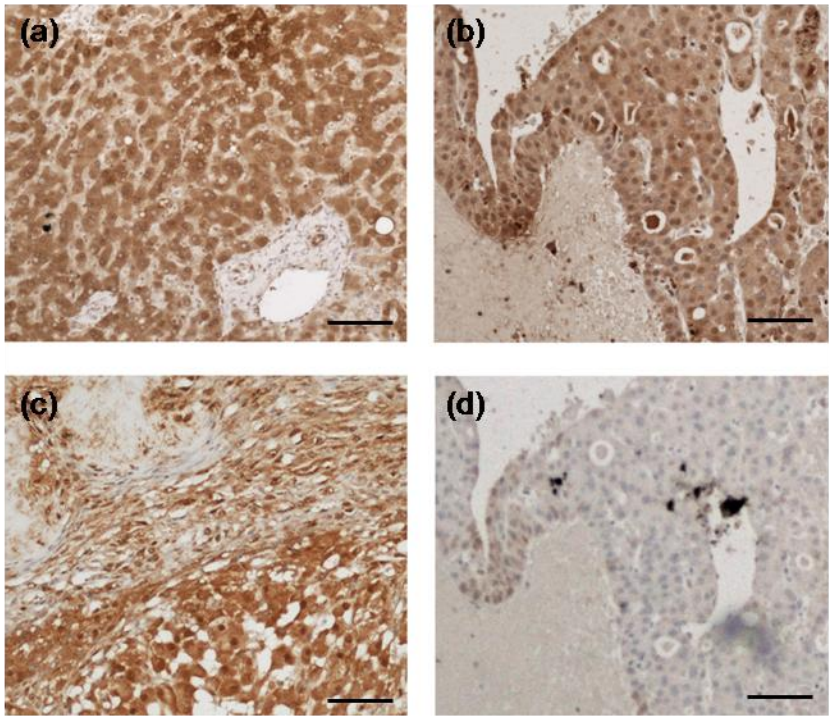

Fig.4 Immunohistochemistry of inhibin βB subunit in HCC patient's liver sections Strong inhibin βB is detectable in the adjacent non-tumour (a) and in the tumor area (b). A tissue section of ovarian carcinoma was used as positive conrol (c). The primary antibody was omitted on this HCC tumor section (d). Bars represent 250 µm.

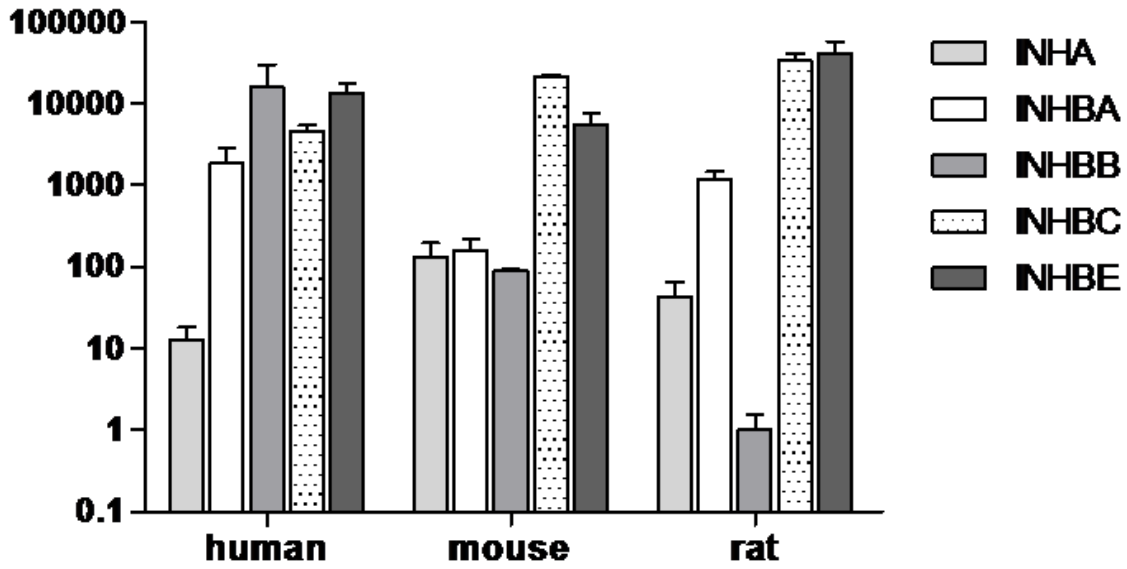

Fig.5 Species comparison of inhibin gene family expression in liver tissues
The lowest expressing gene, rat INHBB (C_t 37.5), was used as calibrator and fold expression ratios for all others were calculated as described in material and methods. Expression ratios are depicted as following, INHA (ligth grey bars), INHBA (open bars), INHBB (dark grey bars), INHBC (pointed bars) and INHBE (black bars).

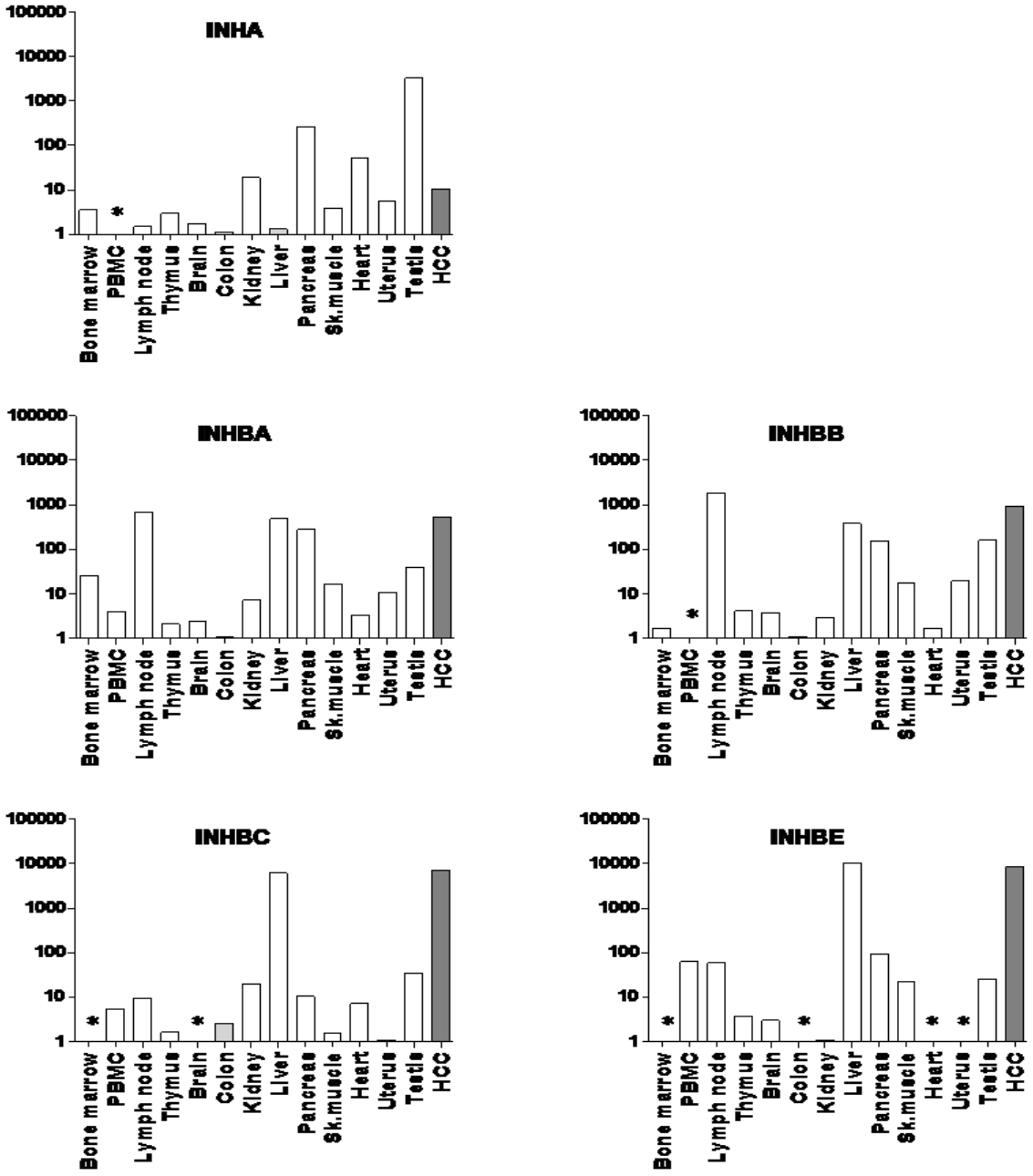

Fig.6 Expression of inhibin subunits in human tissues

C_t values detected in the distinct tissues, rounded to the nearest x.0 or x.5, ranged from 24.0 to 36.0 for INHA, from 27.5 to 37.0 for INHBA, from 24.5 to 35.5 for INHBB, from 26.0 to 38.5 for INHBC and from 24.5 to 36.5 for INHBE. Two and four tissues were below detection limit for INHBC and INHBE, respectively. The lowest expressing tissue was used as calibrator, marked by an arrow, and fold expression ratios for all other tissues were calculated as described in material and methods. Disease-free liver expression levels are marked with a light grey bar, HCC expression levels with dark grey bar. Tissues with undetectable expression are marked with an asterisk.

Discussion

The involvement of TGFβ superfamily members in hepatocellular carcinogenesis is well established (Rodgarkia-Dara et al., 2006; Teicher, 2001), but the individual players and their complex molecular mechanisms of action are still largely unknown. In the present study, we describe for the first time the expression of the complete inhibin gene family in human liver and in hepatocellular carcinoma.

Inhibin and activin A were discovered as proteins regulating the release of follicle stimulating hormone (FSH) (Ling et al., 1986; Vale et al., 1986). Since then activin A has become the best characterised member of the inhibin/activin protein family regarding its physiological functions and the modes of its action. Besides its function in the pituitary, it is involved in many physiological processes, including embryonic development, erythroid differentiation, fibrosis, inflammation, proliferation and apoptosis regulation as reviewed in (Rodgarkia-Dara et al., 2006). One of the first studies about apoptosis induction by activin A was performed in rat livers and primary hepatocytes (Schwall et al., 1993). In the following years a series of studies revealed a potent growth inhibitory role for activin A in the liver (Hully et al., 1994; Kogure et al., 1995; Yasuda et al., 1993).

Unexpectedly, in human HCC the expression of subunit was not found to be downregulated. One possible explanation is the mode of regulation of activin A, which is known to be antagonised by different extracellular factors, including follistatin (Esch et al., 1987; Mashima et al., 1995), fstl3 (Tsuchida et al., 2000) and cripto (Gray et al., 2003). In fact, our group recently described a strong upregulation of follistatin expression in rat liver tumours and human HCC (Grusch et al., 2006). In addition, inhibin A, the heterodimer of the inhibin α and inhibin βA subunit, was found to antagonize the anti-proliferative function of activin A in the liver by competitive binding to activin receptors (Xu et al., 1995). In the inhibin α nulled mouse model, activin A serum levels were enhanced more than 10-fold, leading to apoptosis and necrosis in the liver (Matzuk et al., 1994).

The present results revealed a 9-fold increase in expression of inhibin α mRNA and in 2 of 16 patients even a 1000-fold increase compared to disease-free liver as well as an enhanced expression of the protein (Table 1, Fig.3, Fig.4). This pronounced upregulation of the α subunit may lead to a shift from producing activin A protein to producing inhibin A protein, thereby blocking the anti-proliferative activin A signal and providing growth advantage for tumour cells.

Similarly, the pronounced the inhibin βB subunit expression in HCC samples may cause a shift from activin A to activin AB and/or activin B protein production, which was reported to have less or no effect on hepatocyte proliferation (Niimi et al., 2002). In conclusion, the enhanced expression of inhibin and activin B may serve as alternative or additional

regulators besides follistatin, fstl3 and cripto, overriding the antiproliferative and proapoptotic action of activin A.

Our data further reveal a discrepancy of the inhibin βB mRNA expression between rodent and human liver. This is not due to an altered detection method, because we confirmed the Real-Time PCR results gained in earlier studies reporting weak or undetectable rat INHBB expression levels in Northern or RNA protection assays (De Bleser et al., 1997; Kobayashi et al., 2000; Vejda et al., 2002a). This human-rodent discrepancy may be due to genetic differences or to environmental conditions, with laboratory rodents being largely protected from external noxa. In a recent study, expression was strongly, but transiently enhanced in mice treated in vivo with lipopolysaccharide, mimicking bacterial infection (Jones et al., 2007).

In fact expression of βB in the disease-free human liver samples was as high as in testis (Fig. 4), one of the organs known to show high expression (Roberts, 1997; Vejda et al., 2002a). Only few data about βB expression levels in human liver are available. In human fetal liver βB mRNA was detected by Northern blot assay to be similarly expressed as βA, and generally classified as low expressed in comparison to fetal neural tissues and testis (Tuuri et al., 1994). Sjoholm et al. detected very low levels of βB by Real-Time PCR after normalizing to cyclophilinA (Sjoholm et al., 2006), whereas in a large scale gene expression profiling study on the same samples (Su et al., 2002) liver βB levels are higher than in pituitary gland, but lower than in testis (http://biogps.gnf.org/ ; INHBB; 38545_at Original Affymetrix Annotation).

The balance between the distinct inhibin subunits seems to be crucial for homeostasis; in several tumours deregulation of one or more subunits is a common event. In adrenocortical neoplasms βA expression was elevated in carcinomas (Hofland et al., 2006). βA (Hofland et al., 2007) and βB (Salmenkivi et al., 2001) mRNA was upregulated in malignant but not in benign pheochromocytomas. In malignant endometrial tissues βB was found to be upregulated compared to normal endometrium (Worbs et al., 2007). Overexpression of the α subunit is pronounced in gonadal stromal tumors and even used as immunohistochemical tumour marker (Ciris et al., 2004; Fine and Li, 2003; Fuller et al., 1999). Likewise, elevated serum levels of the inhibin α subunit peptide were found in granulosa cell tumour patients (Burger et al., 2001). Although this subunit is described as restrictively expressed in pituary, adrenal gland and in gonadal tissue, a report about a single case of expression in hepatocellular diseases are available (Vrettou et al., 2005), whereas in another histological studies no cases of inhibin α protein in liver were detected (Lau et al., 2002; Renshaw and Granter, 1998). Also an early histological study in HCC claimed overexpression of inhibin protein (McCluggage et al., 1997), but this finding was disproved a few years later as staining artefact caused by endogenous biotin (Iezzoni et al., 1999). In our hands no cross reactivity

with biotin occured as proven by incubation without antibodies (Fig.4 and 5) and the inhibin α staining in the tumor areas but not in adjacent tissue corresponds to strongly elevated mRNA levels.

The expression levels of all four β subunits is lower in tumor-adjacent liver tissue compared to disease-free samples and increase again in tumor samples (Table 1). A similar pattern was detected for the α subunit in benign and malignant pheochromocytomas in comparison to the normal cortex (Hofland et al., 2007). This drop may be caused by the altered cellular composition in fibrotic and cirrhotic tissue as the β subunits are mainly expressed by parenchymal cells but not by mesenchymal cells. Possibly, these results may reflect a role for the β subunits and the resulting proteins in events associated with fibrosis and cirrhosis rather than with malignancy.

In conclusion, this study suggests important regulatory functions of inhibin subunits in the liver and also during hepatocarcinogenesis.

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2. Manuscript II in preparation

TGFβ family member activin Cs signaling mechanisms differ between primary human hepatocytes and hepatoma cell lines.

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Abstract

Hepatocellular carcinoma is among the leading causes of cancer deaths; however, the underlying molecular pathology is not well understood. There is increasing evidence that activins, belonging to the mammalian transforming growth factor-β (TGFβ) family, are involved in the process of liver carcinogenesis. Like other members of TGFβ family proteins activins elicit their actions through transmembrane serine/threonine kinase receptors and Smad proteins to regulate cellular functions. Whereas the signaling cascade for activin A is well investigated, receptors and downstream signaling for activins containing βC or βE subunits are hardly elucidated so far. Additional complexity is caused through cross talk of the canonical Smad pathway with MAPK pathways, and recent data even suggest signaling via MAPKs in a Smad independent way.

In this study we show for the first time that activin C signaling differs between primary human hepatocytes and hepatoma cell lines, as activin C stimulates phosphorylation of ERK in cells without basal Smad phosphorylation. The second part of this paper focuses on the consequences of differing signaling modes on viability and apoptosis. We report that activin C is able to induce apoptosis by ERK phosphorylation in a Caspase 3 independent way.

Introduction

In many parts of the world hepatocellular carcinoma is among the leading causes of cancer deaths (Caldwell and Park, 2009; Gomaa et al, 2008). Usually chronic tissue inflammation leading to fibrosis and cirrhosis are its precursors (Drucker et al., 2006; Grasl-Kraupp et al., 2000; Herzer et al., 2007; Seitz and Stickel, 2006), and it is characterized by deregulated proliferation and apoptosis of hepatocytes. However, the underlying molecular pathology is not well understood (Laurent-Puig and Zucman-Rossi, 2006; Macheiner et al., 2006; Strand et al., 1996). There is increasing evidence that activins, belonging to the mammalian transforming growth factor-β (TGFβ) family, are involved in the process of liver inflammation and carcinogenesis (Risbridger et al., 2001). They consist of two disulfide-linked β subunits, whereas four mammalian activin β subunits - βA, βB, βC, βE - have been identified. Tissue expression analyses revealed that βA and βB are expressed in most tissues and cell types (Tuuri et al 1994, Vejda et al. 2002), whereas βC and βE are predominantly expressed in the liver, with moderate levels in few other organs (Vejda et al. 2002, Hashimoto et al. 2002, Loveland et al. 1996, Gold et al. 2004, Thomas et al. 1998, Mellor et al. 2000).

Like other members of the TGFβ family activins elicit their actions through heterodimeric complexes of type I and type II serine/threonine kinase receptors (Attisano et al., 1996; Derynck and Feng, 1997; Mathews, 1994). Upon activin binding to type II receptor, which is a constitutively active kinase, the type I receptor is recruited, phosphorylated and therefore activated (Attisano et al., 1996; Zimmerman and Mathews, 1996). Two type II receptors for activin A (ActRII and ActRI) have been identified (Attisano et al., 1992; Carcamo et al., 1994; Mathews and Vale, 1991; Tsuchida et al., 2004). Its main type I receptor is ALK4, also known as ActRIB, whereas activin B and AB prefer Alk7 (Tsuchida et al., 2004). Signals from TGFβ and activin A receptors I are transmitted to the transcription factors Smad2 and Smad3, which are recruited to the receptor complex and activated by phosphorylation (Siegel and Massague, 2003). The phosphorylated R-Smad and the Co-Smad 4 form complexes that translocate to the nucleus to activate the transcription of target genes. The inhibitory Smads 6 and 7 counteract the signal-transducing Smads by preventing the phosphorylation of R-Smads. About receptors and downstream signaling for activins containing βC or βE subunits hardly anything is known so far.

Recent evidence suggest that activin A, similar to TGFβ, may not only depend on the canonical Smad signaling, in fact also mitogen- and stress- activated protein kinases (MAPK/SAPK) contribute to modulation and execution of its signaling (Frey and Mulder, 1997; Xiao et al., 2002). Actually, several reports show cell type- and condition-dependent regulation of alternative signaling pathways including MAP kinases like ERK, JNK or p38. These pathways may be simultaneously activated with the Smad pathway to converge on the

target genes resulting in an enhancement of the Smad-dependent response, some may directly regulate Smad activation, but others might induce responses unrelated to transcription. There are several biochemical links between the TGFβ receptor and MAPK pathways, for example the proapototic adaptor protein Daxx or the TGFβ-activated kinase 1 (TAK1) (de Caestecker, 2004; Derynck and Zhang, 2003; Massague et al., 2000; Moustakas and Heldin, 2005).

Thus, the balance between direct activation of Smads and MAPK pathways often defines cellular responses to TGFβ family members. One very important biological mechanism in which these pathways converge is apoptosis. It is an evolutionary conserved and controlled process essential for normal development, tissue homeostasis, host defense, and suppression of oncogenes through programmed death of single cells without affecting the whole tissue. The morphological features characterizing apoptotic cell death include nuclear condensation, cell shrinkage, and plasma membrane blebbing resulting in apoptotic bodies (Arends and Wyllie, 1991; Kerr et al., 1972; Taylor et al., 2008). In the liver apoptosis fine tunes the complex system regulating liver function. One important feature of the liver is its enormous regeneration capacity after cell loss through physical, infectious or hepatotoxic injury (Kren et al., 1997). Disturbance of the balance between cell proliferation and apoptosis can promote proliferation and transformation of cells therefore leading to chronic hepatitis, cirrhosis and liver cancer (Leevy, 1998; Schattenberg et al., 2006). Additionally, genetically altered cells no longer extinguished through apoptosis contribute to hepatocarcinogenesis.

TGFβ appears to be central in all the multiple events of proliferation and apoptosis, displaying growth stimulatory effects for cells of mesenchymal origin, but growth-inhibitory ones on cells of epithelial origin. In the liver TGFβ is one of the few identified negative regulators of liver cell mass, acting by induction of apoptosis (Oberhammer et al., 1991; Whitman, 1998). Amongst the activin subfamily only the well investigated activin A has been implicated in liver growth regulation by its capability to inhibit mitogen induced DNA synthesis (Yasuda et al., 1993) and to induce apoptosis in vivo and in vitro (Chen et al., 2000; Schwall et al., 1993). Data on activin C are rather conflicting as Vejda et al. report the potential to induce apoptosis like activin A and TGFβ, whereas Wada et al. show stimulated DNA synthesis and increased numbers of cells after activin C treatment (Vejda et al., 2003; Wada et al., 2004b). However, the molecular mechanisms underlying activin induced growth regulation remain poorly understood.

In the present study we compared human primary hepatocytes with the well defined hepatocellular carcinoma cell model systems HepG2 and Hep3b regarding their signaling in response to activin A, B and C treatment. HepG2 are known to still share more characteristics with primary hepatocytes than Hep3b, which express several mesenchymal proteins indicative for EMT (Slany et al., 2009). Thus, by using these three different cell

types, we address different phases of hepatocarcinogenesis. Furthermore this study focuses on consequences of the activated signaling pathways regarding viability and apoptosis in those different liver derived cells.

Material and Methods

Cell culture

Human liver cell lines HepG2 and Hep3 from ATCC were maintained in RPMI 1640 (Gibco) supplemented with penicillin/streptomycin (100 U/ml; Sigma) and 10% FCS (PAA Laboratories). Primary human hepatocytes were isolated and cultivated in serumfree medium as previously described (Weiss et al., 2002; Weiss et al., 2003). In brief, tissue samples from liver resection were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research), with the informed patient's consent approved by the local ethical committee of the University of Regensburg. Cells were cultivated in serumfree Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 7ng/ml glucagon, 0.5U/ml insulin, 8µg/ml hydrocortison, 100 U/ml penicillin/streptomycin and 2nM L-glutamine. Treatment was performed 72 hours after plating. Primary mouse hepatocytes were obtained from wild type and knockout mice provided by Heinrich Schrewe (Max Planck Institute for Molecular Genetics, Berlin, Germany). Isolation and cultivation was performed as recently described by van Zijl et al. (van Zijl et al., 2009). All of the culture incubations were carried out at 37°C in humidified atmosphere and 5% CO_2.

RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Cell lines were harvested for RNA preparation during logarithmic growth and directly lysed in TRIzol. RNA concentration and ratio 260/280nm were determined photometrically on GenQuant (Pharmacia).

Reverse Transcription and Quantitative Real-Time PCR

Two micrograms of total RNA were reverse transcribed with MMLV RevertAid (Fermentas) resulting in 100µL cDNA solution. Two microliters cDNA were used as a template for each PCR. Real-Time analysis was performed with TaqMan® Universal PCR Master Mix (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) running the following protocol: initial 50°C 2min and 95°C 10min, and 40 repeats of 95°C 15 sec, 60°C 1 min. Taqman assays for human βA (INHBA; Hs00170103_m1), βB (INHBB; Hs00173582_m1), βC (INHBC; Hs00173745_m1) β2-microglobulin (B2M; Hs99999907_m1) and GAPDH (GAPDH; Hs02758991_g1) were obtained from Applied Biosystems. All samples were analyzed in duplicate in at least two independent Real Time PCR runs.

Western blotting

Cells were treated with 10ng/ml recombinant human activin A, activin B, activin C and TGFβ (R&D Systems) in serumfree RPMI (Gibco) for 30min. 2.5 ng/ml FGF2 served as positive control for ERK phosphorylation, 25 ng/ml anisomycin as positive control for p38 phosphorylation. They were then solubilized in a lysis buffer containing 50mM TrisCl pH 7.4, 500mM NaCl, 1% NP-400, 5% Na-Deoxycholat, 0.1% SDS0, 0.5% NaN_3, 100mM Na_3VO_4 and complete (Roche). Proteins were separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Amersham Biosciences). Blots were incubated with blocking solution (10% low fat milk, 0.05% Tween 20 in PBS) for 20min. The following primary antibodies were used at a dilution of 1:1000: Smad2/3 (Cell Signaling Technology Inc.), ERK, p38 plus the respective ones for the phosphorylated forms, and βActin (all from Sigma). Subsequently, corresponding secondary antibodies (Calbiochem) were applied, and visualization was performed with an enhanced chemiluminescense detection system (Thermo Fisher Scientific) according to the manufacturer's instructions. The blots were on the one hand exposed to X-ray film, on the other hand detection was performed using a Chemi Smart 5001 (Peqlab). Quantification was performed using Image Quant.

Luciferase assay

HepG2 and Hep3b cultured in 24-well plates were transiently transfected with 1µg of the indicated constructs using Fugene 6 (Roche) transfection reagent following manufacturer's protocol. To correct for transfection efficacy, 100ng renilla luciferase (pRL-CMV, Promega) were cotransfected. pGL3 vector DNA was used to keep the total amount of DNA in all samples constant. On the next day cells were washed with PBS and treated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in serum free RPMI 1640 for 6 hours. Luciferase activities were quantified using Dual-Luciferase Assay (Thermo Fisher Scientific) according to manufacturer's instructions. Emitted luminescence was measured using a Labsystems Luminoscan. Firefly luciferase activity was normalized for renilla luciferase activity.

Luciferase reporter gene constructs.

For intracellular signaling of TGFβ, a CAGA luciferase construct, consisting of 12 Smad3/4 binding sequences (CAGA boxes) and the luciferase-coding sequence, was used. The CAGA boxes confer TGFβ stimulation to a heterologous promoter reporter construct, whose activity depends on binding of activated Smad3/4 transcription factor complexes (Dennler et al., 1998). Additionally, a 3TP-Luc plasmid, which besides the luciferase coding sequence consists of plasminogen activator inhibitor 1 (PAI-1) gene promoter containing three repeats

of a TGFβ–responsive element and Smad2/3, was used (Wrana et al., 1992). Plasmids were kindly provided by Wolfgang Mikulits (Cancer Research Institute, Vienna, Austria). To assess Smad independent signaling another cis-acting construct was applied: an AP1 reporter for measuring the modulation of the JNK/AP1 pathway. The other constructs for measuring Smad independent signaling all were trans-acting constructs which had to be transfected together with a pFR-Luc reporter plasmid: a pFA2-ELK1 that is designed to measure transcriptional activity of ELK1, the nuclear target for the MAPK/ERK signaling cascade, and a pFA-CHOP construct indicating the p38 activation. Plasmids were kindly provided by Johannes Schmid (Centre for Biomolecular Medicine and Pharmacology, Medical University Vienna, Austria).

Immunofluorescence microscopy

Cells were seeded to glass slides, and incubated with 10ng/ml recombinant human activin C (R&D Systems) for 6 hours. Cells were fixed in 4% formaldehyde (Sigma) in PBS for 10 min at room temperature. After blocking for 30 min (1% PBS, 0.5% Tween 20, 0.2% fish gelatin) at room temperature, 200 ml of a 1:100–1:300 diluted Smad2/3 primary antibody (BD Transduction Laboratories) was applied and incubated overnight at 4°C. The corresponding TRITC (Tetramethylrhodamine Iso-Thiocynate) labeled secondary antibody was diluted in PBS Tween 20 containing 0.2% gelatin. 200 ml of the mixture per slide were incubated for 2 hours. Nucleic staining was performed using a 1:1000 dilution of a 5mg/ml DAPI stock solution (4',6-Diamidin-2'-phenylindol- dihydrochlorid, Roche Diagnostic). After incubation for 5min, the slides were washed with PBS and covered with mounting medium (Sigma). Imaging was performed by fluorescence microscopy using a Nikon Eclipse TE 300 microscope equipped with a Nikon Digital Camera DS.

MTT-Assay

Cell proliferation/Viability was assessed based on mitochondria number by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. A total of $3x10^3$ HepG2, respectively $0.8x10^3$ Hep3b cells were added to each well of a 96-well microtiter plate and allowed to attach overnight. Cells were incubated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ (R&D Systems) in serumfree RPMI 1640 for 48 hours. MTT (Fluka) was dissolved to a concentration of 5 mg/ml in PBS and 30µl of this MTT stock solution were added per well. Incubation at 37°C took approximately 30min. Cells were then washed once with PBS and 100µl DMSO (Sigma) were added to each well to lyse the cells. Absorbance was immediately measured at 550nm using an automatic micro-plate reader (Synergy HT).

Analysis of apoptosis by flow cytometry

$2x10^5$ HepG2 and $1×10^5$ Hep3b cells were seeded in six-well plates and allowed to attach overnight. PHH were used 72 hours after isolation. Transient transfection with 1µg wildtype ERK respectively the dominant-negative mutant Y185F (Robbins et al., 1993) was performed using Fugene 6 (Roche) transfection reagent following manufacturer's protocol. Three wells were incubated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ (R&D Systems), harvested 48 h later through trypsinization, and washed twice with cold PBS. Cells were centrifuged at 3000 rpm for 5 min and the pellet was resuspended in PBS and incubated with 10µl of propidium iodide (30µg/ml, Invitrogen) for 15 min at room temperature in the dark. A Caspase 3 Assay to detect cleaved Caspases as indicator for apoptosis was performed according to manufacturer's introduction (BioVision). Analysis was carried out in a Becton Dickinson FACSCalibur system using CELLQuest software. 1 µg GFP served as transfection control and only cells positive for GFP and PI were analyzed.

Statistical analysis

All experiments were repeated at least three times. Values are expressed as mean ± SD, with $p<0.05$ considered significant. Statistical differences were determined using the one sample t-test (GraphPad Prism 5 package).

Results

Inhibin gene expression in primary human hepatocytes and human hepatoma cell lines

Inhibin gene expression profile was investigated by quantitative Real Time PCR analyses to reveal differences, respectively similarities, between primary human hepatocytes (PHH) and the two human hepatoma cell lines HepG2 and Hep3b. The expression of inhibin subunits in PHHs, HepG2 and Hep3b are depicted in Fig.1.

B2M and GAPDH were analysed as reference genes. In contrast to HCCs and tumor/non-tumor tissues were both housekeeping genes (HKG) exhibit differences in expression levels and were considered as regulated (Frost et al., manuscript in preparation and manuscript I), no such regulation was observed for B2M and the reference HKG GAPDH comparing PHH, HepG2 and Hep3b. C_t levels were consistent not only within one cell type but also between the different cell types, ranging from 19 to 21. Thus, expression data were simply normalised to the expression of B2M according to commonly followed procedures.

In PHH all three β subunits are expressed at similar levels, with the liver specific inhibin βC as the most prominent one. In HepG2 inhibin βC and βB still are expressed as high as in PHH, whereas inhibin βA is expressed about 2^{10} times lower. In Hep3b all three subunits are expressed at comparable but low levels. Overall, PHH and HepG2 cells share similarities regarding Inhβ mRNA expression. The low inhibin βA expression in HepG2 corresponds to literature (Chen et al., 2000; Mashima et al., 1995; Vejda et al., 2003). As we consider inhibin βC expression not only as liver but as hepatocyte specific, the loss of expression in Hep3b is in accordance with recent investigations by Slany et al. Their findings demonstrate PHH and HepG2 cells to have substantial similiarity regarding hepatocyte specific protein expression, whereas Hep3b have lost this features (Slany et al., 2009).

Activin C does not primarily signal via Smad2/3 in PHH and HepG2

To determine if activins similar to TGFβ signal via Smad2/3 and whether there are differences in signaling between primary hepatocytes and hepatoma cells, we addressed the transductions mechanism by three different methods: Western blotting, luciferase assay and immunofluorescence. As depicted in Fig.2A and 2B activin A, activin B and TGFβ clearly induced Smad2/3 signaling in PHH and HepG2, whereas activin C had no effect. Hep3b differed from the other cells examined, as activated Smad was already detected in untreated cells. Again activin C did not change the phosphorylation status, but also activin A and B had only minor effects on the phosphorylation level of Smad2/3. As mRNA analyses revealed high expression of InhβC in PHH, we were interested in the influence of endogenous activin C on the underlying pathway. This was done by comparing the basal smad phosphorylation

status of wildtype and knockout mouse primary hepatocytes as well as their reaction to added recombinant activins. However, endogenous InhβC expression does not have any obvious influence on basal signaling or signaling induced by exogenous activins as signaling pattern in wild type and knockout primary hepatocytes resemble each other (Fig. 2B). To confirm the results gained by western blot analysis we used a transcriptional activation assay. The reporter construct used in this study is CAGA, which contains a luciferase gene under the control of a synthetic promoter that has been widely used in TGFβ signaling studies (Dennler et al., 1998). Transfection dependent luciferase assay were performed in the cell lines only, because primary hepatocytes hardly ingest any foreign DNA. Luciferase values in PHH transfected with the CAGA construct were below the limit of detection (data not shown). Thus, HepG2 exemplified for PHH due to their similarities shown before. In HepG2 cells, the reporter construct had very low basal activity, but was significantly stimulated more than 130-fold after treatment with 10ng/ml activin A, activin B and TGFβ for 6 hours. According to western blot results activin C could not induce luciferase expression. Also in Hep3b luciferase assay clearly resemble the results of the western blot analysis: basal level was rather high but induction of CAGA driven luciferase activity was rather low in respond to the activins, whereas TGFβ stimulation had the only significant effect. Results obtained with a second Smad responsive reporter construct, namely 3TP-Luc, proved these findings (data not shown). Fluorescence microscopy visualized the differences between HepG2 and Hep3b regarding their basal Smad phosphorylation status: whereas no Smad signal is detected neither in the nucleus of untreated nor in activin C treated HepG2, the fluorescence is equally strong in the nucleus of Hep3b no matter if treated with activin C or not.

Thus, analysis of the Smad signaling pathway unfold different basal states of Smad phosphorylation for PHH and HepG2 on the one hand and again the already more dedifferentiated Hep3b on the other hand. However, activin C does not activate Smad2/3 signaling in any of the hepatoderived cells.

Activin C induces Smad independent signaling via ERK in PHH and HepG2

As activin C did not induce Smad signaling, we explored its ability to activate members of the Smad independent signaling cascade. Thus, activin C clearly induced ERK phosphorylation in PHH as well as in HepG2. As no signaling via Erk was generated through activin C in Hep3b cells their exceptional position was confirmed (Fig.3A). Phosphorylation of p38 in contrast was induced by activin B and activin C in PHH, and by activin A and activin B in HepG2. In Hep3b quantification falsifies the outcome as phosphorylation seems to stabilize total protein. Considering phosphorylated p38 activin A, B and C may cause an enhancement. No phosphorylation of JNK was induced upon activin treatment (data not

shown). To prove the overall responsitivity of PHH, HepG2 and Hep3b for ERK and p38 phosphorylation we incubated them with the known positive controls FGF2, respectively anisomycin. All three were able to phosphorylate ERK respectively p38 in response to the positive controls (Fig.3B).

We confirmed results regarding activin C induced ERK phosphorylation by transactivation reporter assay. HepG2 and Hep3b were transiently transfected with a reporter construct designed to measure transcriptional activity of ELK1, the nuclear target for the MAPK/ERK signaling cascade. As depicted in Fig.3C ERK cotransfection was necessary for activation of the ELK1 construct, thus also Hep3b generate a luciferase signal. Addition of activin C enhanced the reporter activity in HepG2, but decreased it in Hep3b. Again, in Hep3b the basal level was about 3-fold higher than in HepG2 cells (Fig.3C first bars). No activation of JNK or p38 was seen in luciferase assay (data not shown).

These result indicate, that activin C does not necessarily utilize the canonical Smad pathway in liver derived cells. For the first time we show that the ERK pathway is activated by activin C only in cells with unphosphorylated Smad basal level.

Activin Cs ability to induce apoptosis gets lost during dedifferentiation of hepatoma cells

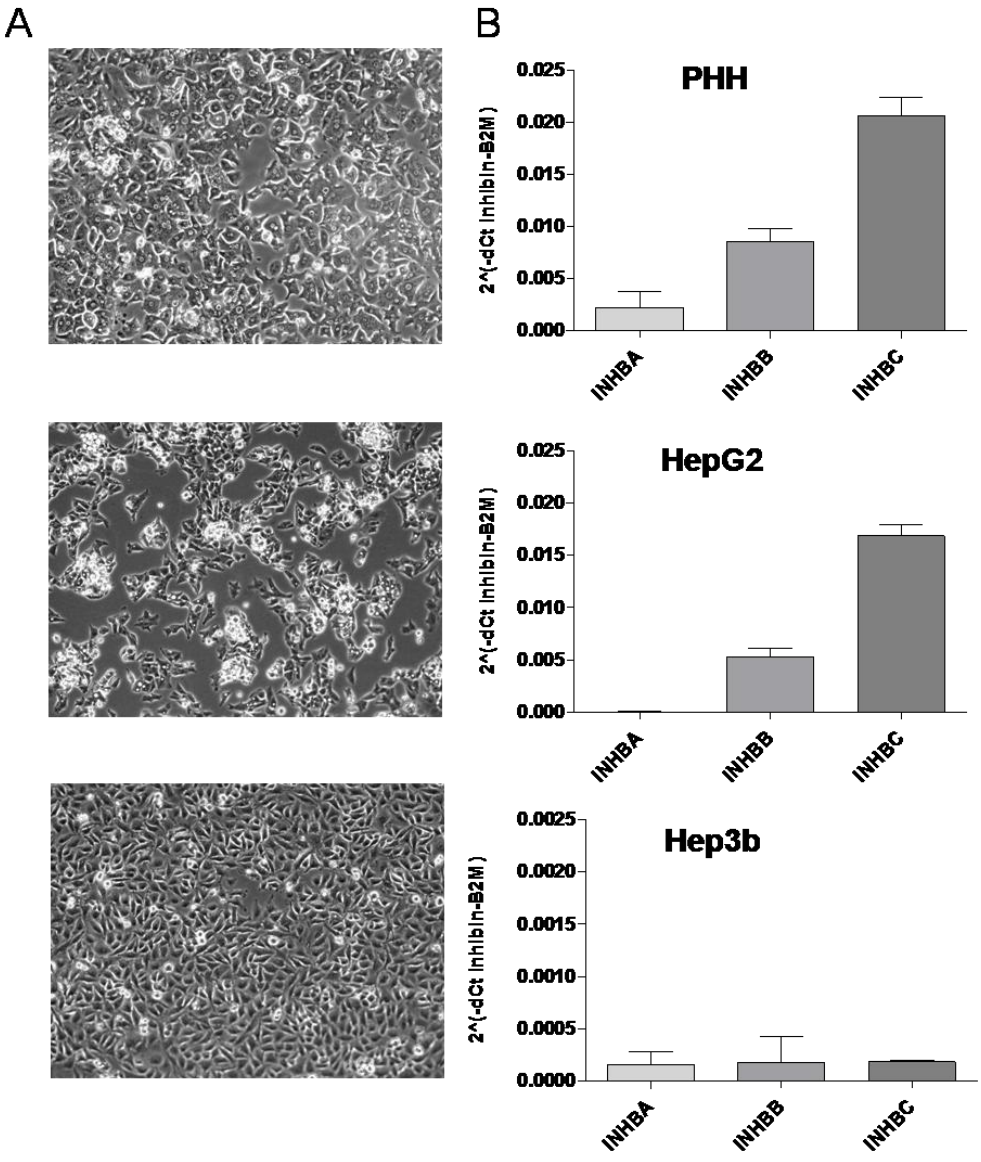
Thus, the question arose whether the altered signaling mechanism has any consequences on the outcome and the biological function of the activin subfamily. TGFβ family members are known to play differential roles in apoptosis, and ERK is a downstream target for cytokines rather promoting survival (Roux and Blenis, 2004). As altered apoptosis is a key mechanism in hepatocarcinogenesis, we focused on the effects of activins on apoptosis and possible differences in the different cell types.

First viability was assessed using a MTT assay (Fig.4). Primary hepatocytes as differentiated cells do hardly proliferate in vitro and therefore are not suitable for this kind of assay. Thus only the two hepatoma cell lines were analyzed. Cisplatin served as viability reducing positive control, with a similar effect in both cell lines. The activins however, caused differential responses. Activin A and B induced a slight yet significant increase of viability in HepG2. Activin C and TGFβ rather had viability decreasing effects in this cell line. In Hep3b activin A also slightly increased viability, whereas activin B, activin C and TGFß significantly decreased viability. FGF2 as strong inducer of ERK phosphorylation (Fig.3B) interestingly reduced viability in HepG2 and Hep3b.

As activin C is discussed contradictory for its apoptosis inducing potential, we analyzed its effect on hepatoderived cells by propidiumiodide staining and Caspase activity assay (Fig.5A and B). Serum deprivation caused an increased of PI positive cells by 2-fold in HepG2 and about 5-fold in Hep3b. In HepG2 activin A and B had no effect, whereas activin C and TGFβ

induced the portion of apoptotic cells about 2.5f-old. These results reflect those obtained by viability assay (Fig.4 left panel). Irrespective to the growth factors added the percentage of PI positive cells did not vary in Hep3b cells (Fig.5A). In PHH PI positive cells increased about 1.5-fold after the addition of all the used growth factors (data not shown). Caspases are proteases specifically induced during apoptosis. Serum deprivation also elevates Caspase 3 activity about 3-fold in HepG2 and 5-fold in Hep3b. All in all the addition of the activins did not further influence Caspase activity in these two cell lines (Fig.5B). These results may indicate Caspase 3 not to be involved. Thus, other Caspases or a Caspase independent mechanism may conduct apoptosis.

As activin C causes a strong ERK phosphorylation signal (Fig.3A) and in parallel induces apoptosis (Fig.5A) in HepG2, we assessed the effect of overexpressed ERK in these cells. Ectopic wildtype ERK increased the number of PI positive cells about 2.5-fold (Fig.6) comparable to treatment with activin C (Fig 5A). A kinase dead ERK mutant had no effect on apoptosis in HepG2 cells. We conclude that the phosphorylation of ERK is responsible for apoptosis induction by activin C. In Hep3b wildtype ERK did not influence apoptosis but the kinase dead mutant induced apoptosis about 2-fold. Apparently, overexpression of the phosphorylation deficient mutant blocks signaling pathways important for survival of cells.



C

	Δct-B2M		
	INHBA	INHBB	INHBC
PHH	8,83	6,87	5,6
HepG2	14,96	7,57	5,89
Hep3b	12,68	12,46	12,42

Fig.1 Inhibin gene expression differs in primary human hepatocytes and human hepatoma cell lines

(A) Morphological differences of PHH, HepG2 and Hep3b cells are displayed. (B) PHH were harvested 72h after plating, HepG2 and Hep3b were harvested during logarithmic growth. mRNA was isolated and the expression levels of InhβA, InhβB and InhβC were determined by quantitative Real Time PCR. mRNA expression is expressed as 2^delta Ct-values (= Ct values of Inhβ – Ct-values of β2-microglobulin). Data were gained as duplicates in at least three independent experiments. They are expressed as means ± SD. (C) Table of delta Ct-values, B2M was used as reference gene.

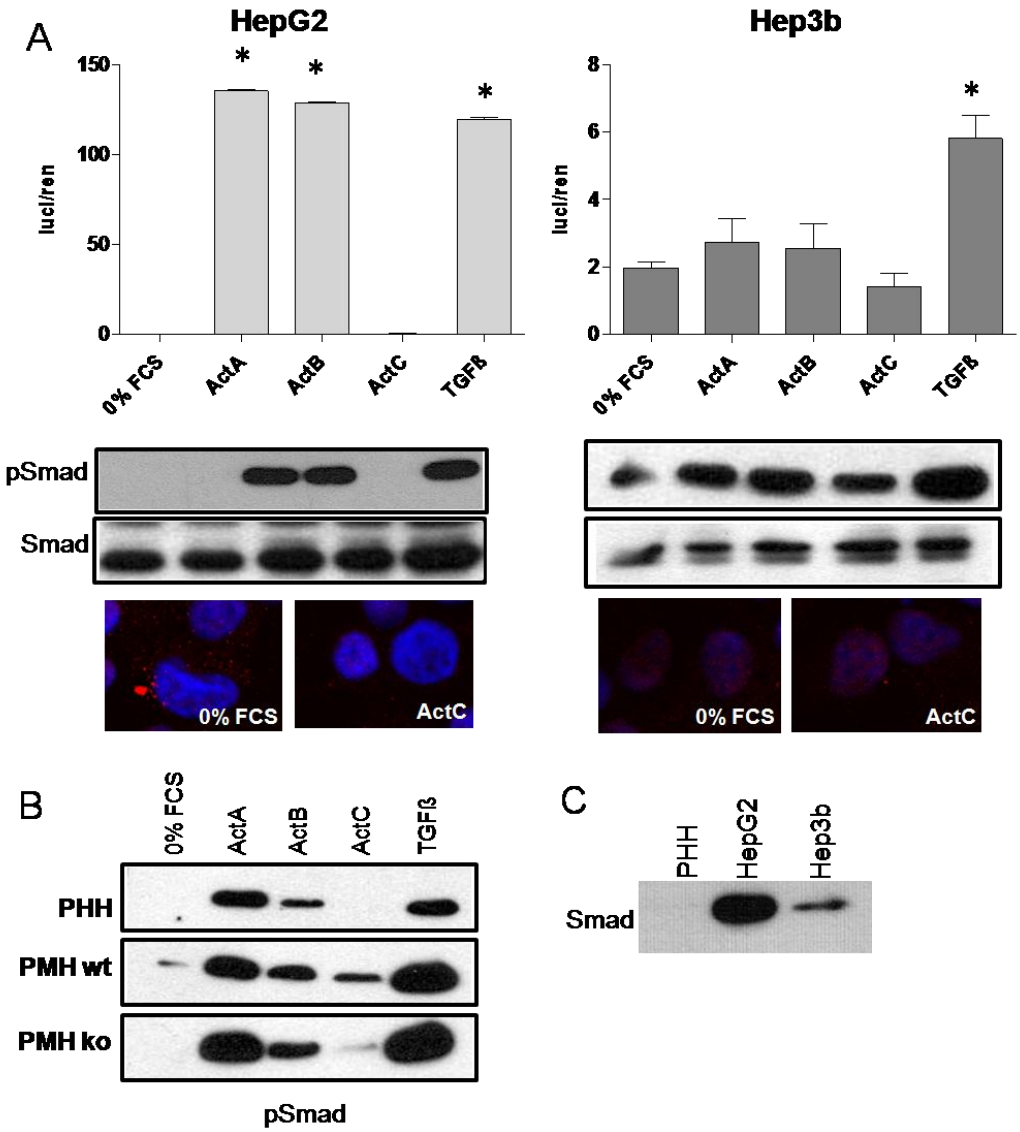


Fig.2 Activin C does not primarily signal via Smad2/3 in PHH and HepG2

(A) HepG2 and Hep3b were transfected with 1µg of the CAGA luciferase construct, consisting of 12 Smad3/4 binding sequences and the luciferase reporter gene. On the next day cells were washed with PBS and treated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in serum free RPMI 1640 for 6 hours. Absolute values for 0% FCS were 0.14 in HepG2 and 1.9 in Hep3b. Values are expressed as firefly luciferase activity normalized to renilla luciferase activity. Data are means ± SD of at least three separate experiments. Significant differences were assessed relativ 0% FCS (* $p<0.05$). For western blot analyses HepG2 and Hep3b were exposed to 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in serum free RPMI 1640 for 30 minutes. Proteins were separated by SDS page and immunoblotted for detection of the phosphorylated and unphosphorylated form of Smad2/3. Experiments were performed at least three times and a representative chemiluminescence blot is pictured. HepG2 and Hep3b cells seeded on glass slides were incubated under serum free conditions with 10ng/ml recombinant human activin C for 6 hours. They were exposed to TRITC labeled Smad2 antibody and nucleus stained with DAPI. (B) Western blot analyses of PHH, wildtype and knockout PMH was performed the same way as for HepG2 and Hep3b. Sensitivity of the pSmad antibody is approximately the same for human and for mouse. (C) Total Smad western blot to assess the basic level of expression in the three different cell lines.

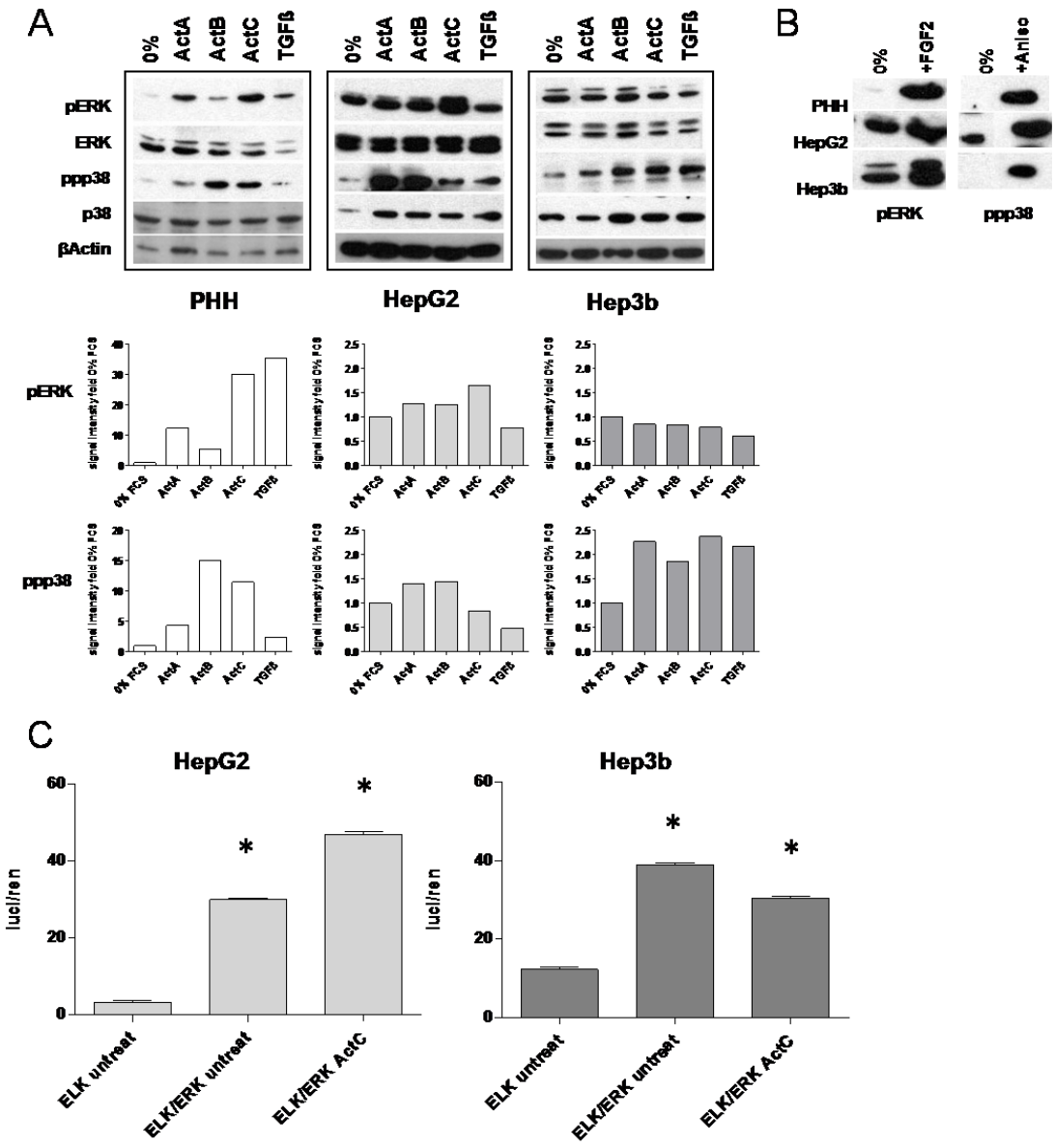


Fig.3 Activin C induces Smad independent signaling via ERK in PHH and HepG2

(A) PHH, HepG2 and Hep3b were exposed to 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in serum free RPMI 1640 for 30 minutes. Proteins were separated by SDS page and immunoblotted for detection of βActin and the phosphorylated and unphosphorylated form of ERK and p38. Phosphorylation levels were evaluated by densitometry, normalized to total protein for equal loading and protein integrity, and expressed as fold of untreated control. A representative chemiluminescence blot and the corresponding densitometric analysis are pictured. (B) All three cell types were incubated with 2.5ng/ml FGF2 and 25 ng/ml Anisomycin a positive phosphorylation control. Western blot analysis was performed as mentioned above (C) HepG2 and Hep3b were transfected with 1µg of the ELK1 transactivation construct, together with the luciferase reporter construct. Additionally 1µg ERK was transfected. On the next day cells were washed with PBS and treated with 10ng/ml recombinant human activin C in serum free RPMI 1640 for 6 hours. Absolute values for ELK untreated were 3.2 in HepG2 and 12.3 in Hep3b. The data are expressed as firefly luciferase activity normalized to renilla luciferase activity and are means ± SD of 3 experiments. Significant differences were assessed relativ ELK untreated (* $p<0.05$).

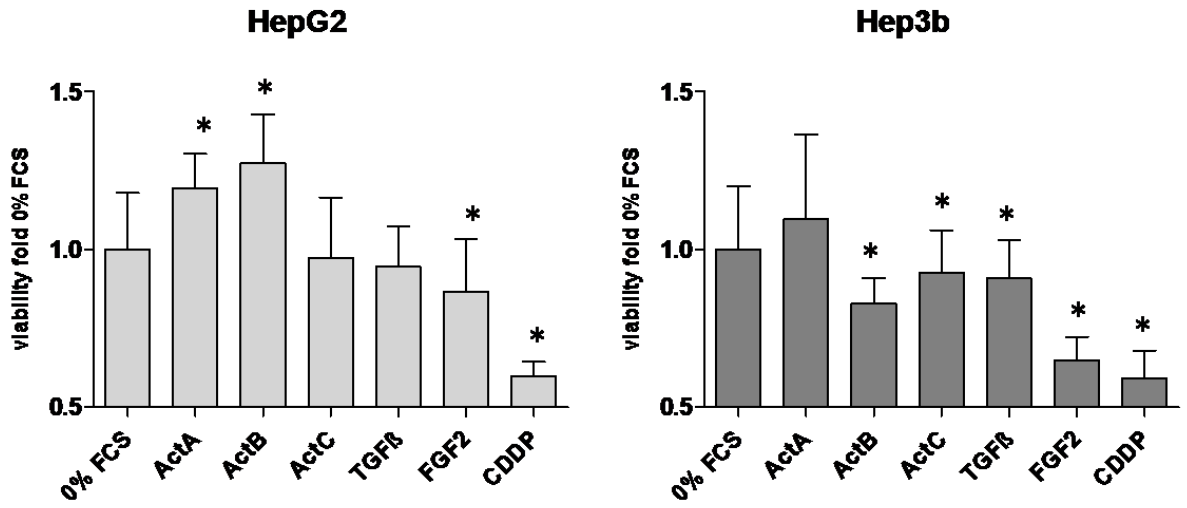


Fig.4 Effect of activins on viability

HepG2 and Hep3b were allowed to attach to 96-well microtiter plates overnight. Cells were exposed to 10ng/ml recombinant human activin A, activin B, activin C or TGFβ under serumfree conditions for 48 hours. Incubation with 30µl of 5mg/ml MTT stock solution at 37°C took approximately 30min. DMSO was used to lyse the cells. Data are expressed as fold 0% FCS. Means ± SD are given from 3 separate experiments with 10 wells per treatment each. Significant differences were assessed relative 0% FCS (* $p<0.05$).

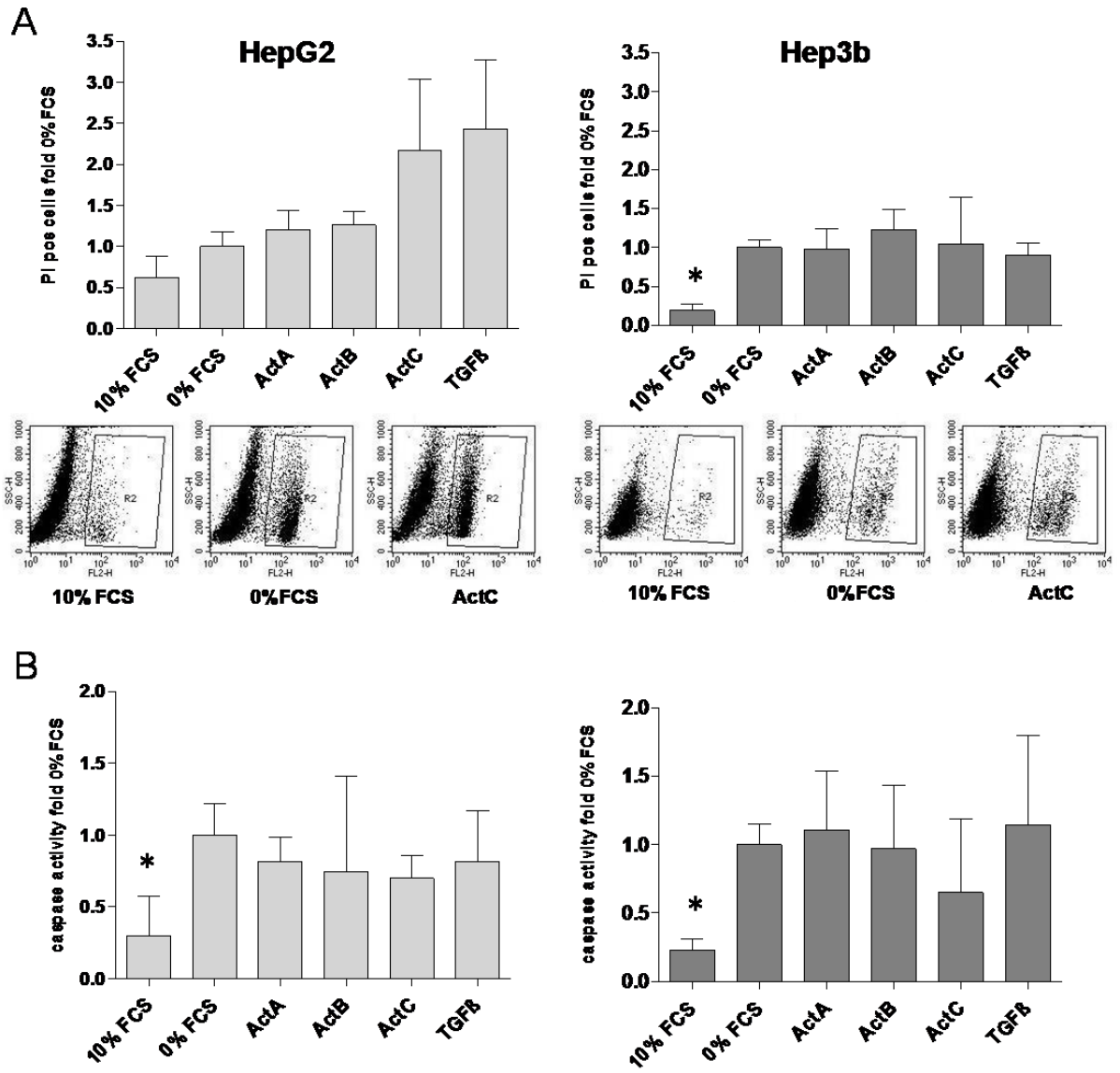


Fig.5 Activin Cs ability to induce apoptosis gets lost during dedifferentiation of hepatoma cells

(A) HepG2 and Hep3b were incubated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ under serumfree conditions for 48 hours. The amount of PI positive cells was determined as described in material and methods. Typical histograms are given for 10% and 0% serum conditions as well as for activin C treatment. (B) Caspase Assay was performed with equally treated cells according to manufacturer's introduction. All data represent results from three independent experiments and are given as means ± SD normalized to 0% FCS. Significant differences were assessed relative 0% FCS (* $p<0.05$).

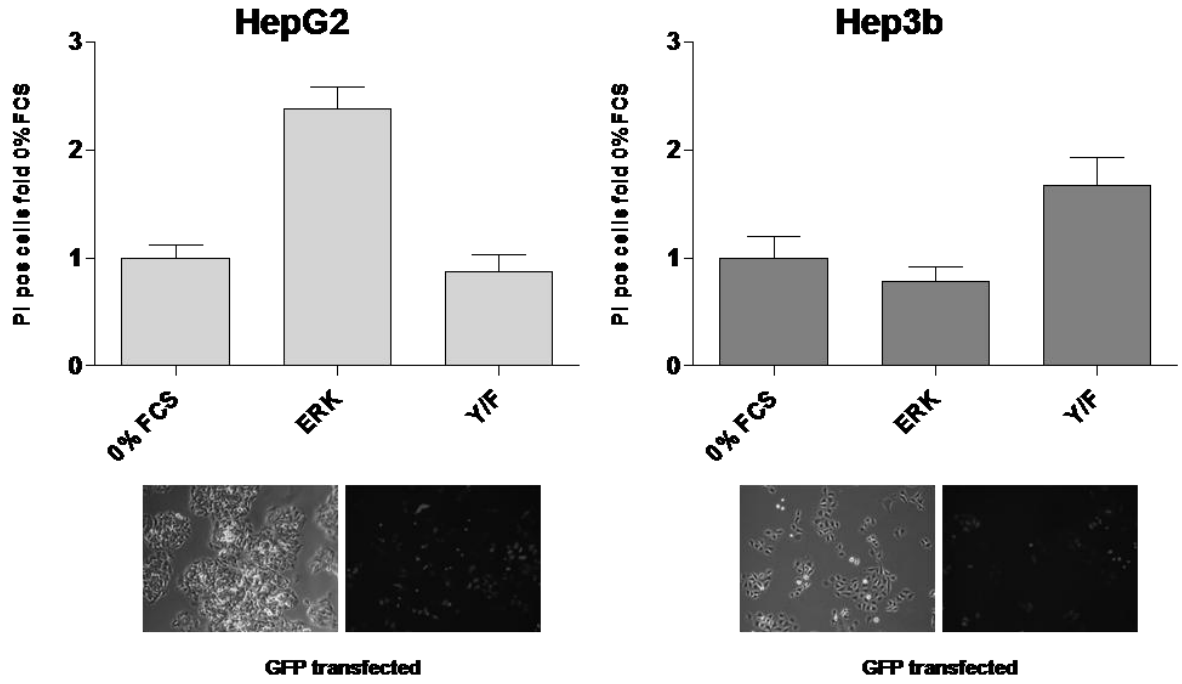


Fig.6 Phosphorylated ERK causes apoptosis in HepG2 but not in Hep3b

HepG2 and Hep3b were transfected with 1µg wildtype ERK respectively its phosphorylation inhibited mutant Y/F. A GFP construct served as transfection control, and only GFP and PI positive cells were analyzed. The amount of PI positive cells was determined by flow cytometry as described in material and methods. Results of three independent experiments are given as means ± SD normalized to 0% FCS.

Discussion

Although activin A and activin B are known to activate Smad signaling via ActRII/ActRIIB and Alk4 respectively Alk7 (Attisano et al., 1992; Carcamo et al., 1994; Mathews and Vale, 1991; Tsuchida et al., 2004), there are only two publications about the signaling mechanisms of activin C (Gold et al., 2009; Mellor et al., 2003). They report that activin C does not signal through Smad2/3 in human prostate tumor cell lines, respectively pituitary cell lines. In the present study we demonstrate that activin C signal transduction does not occur via Smad2/3 in primary human hepatocytes and hepatoma cell lines either. Furthermore, we show for the first time, that activin C signaling differs between primary human hepatocytes and hepatoma cell lines, as activin C stimulates phosphorylation of ERK in cells without basal Smad phosphorylation. Finally, we provide new insights in activin related apoptosis.

We could demonstrate differences in mRNA expression of InhβA, InhβB and InhβC subunits using Real Time RT-PCR. Thus PHH and HepG2 display quiet similar expression pattern as well as amounts of mRNA. Only the expression of InhβA differs between the two cell types, as there is hardly any expression of it in HepG2. This is supported by previous studies (Chen et al., 2000; Mashima et al., 1995; Vejda et al., 2003). Vejda et al however, report no expression of InhβA at all and only very week expression of InhβC in HepG2, which can be explained by the use of the less sensitive RNase protection assay. Their outcomes regarding expression of InhβA and InhβC in primary rat hepatocytes pretty much resemble our results seen in primary human hepatocytes (Vejda et al., 2003). It has to be noticed, that the Inhβ mRNA expression level in primary rat hepatocytes drops by a factor 100 in the first 24 hours after isolation. As we receive primary human hepatocytes only 48 hours after isolation for further analysation we do not no what happens during the previous time period. However, we could detect a decrease of Inhβ mRNA expression approximitally by a factor 10 between 72 hours and 96 hours after isolation, still showing a tendency towards reduced Inhβ mRNA expression (unpublished results). The lack of InhβA subunit and the InhβB subunit expression reported by Chen et al. and thus opposed outcome may be due to different primers used in Real Time RT-PCR, or to a different passage of analyzed cells. Furthermore, this cell line is known to exhibit a rather deranged activin system (Mashima et al., 1995). This dearrangements may indicate an important role for distinct inhibin/activin subunits in liver derived cells. Hep3b show very low but comparable mRNA expression levels of all three subunits. The obvious difference of on the one hand PHHs and HepG2 cell and on the other hand Hep3b is supported by findings of Slany et al. They document HepG2 to share more characteristics with PHH on protein level than with Hep3b (Slany et al., 2009). InhβC is expressed highest in all three cell types analyzed, which is in agreement with its high profile as hepatocyte specific (Vejda et al. 2002, Hashimoto et al. 2002, Loveland et al. 1996, Gold

et al. 2004, Thomas et al. 1998, Mellor et al. 2000). As mentioned above however, Hep3b exhibit very low expression levels for this subunit too. Contrasts and overlaps seen in the expression pattern make them adaquate tools for revealing further differences on other biological levels.

Based on this knowledge, we focussed on the well known signal transducers of the TGFβ family Smad2/3. Neither PHH nor HepG2 showed phosphorylated Smad2/3 in their basal state, whereas it was phosphorylated upon activin A and B treatment. Incubation with activin C however, did not change the basal state of Smad2/3. Hep3b cells in contrast, already showed phosphorylated Smad in the basal state. Activin C again did not change the phosphorylation status, but also activin A and B had only minor effects. Usage of luciferase constructs depending on binding of activated Smad3/4 transcription factor complexes confirmed this output. Hence, PHH/HepG2 and Hep3b do not only differ in the mRNA expression of activins, but also in Smad phosphorylation in the basal state. Most importantly activin C does not activate Smad2/3 signaling in any of the hepatoderived cells. This is in agreement with Gold et al. and Mellor et al. who demonstrate Smad2/3 independent signaling in human prostate tumor cell lines, respectively pituitary cell lines (Gold et al., 2009; Mellor et al., 2003). Activin A is known to signal via ActRII/ActRIIB (Activin receptor II/IIB) and ALK 4 (Activin like kinase 4) and activin B and AB have a preference for ALK7 (Tsuchida et al., 2004), both receptor combinations resulting in Smad2/3 phosphorylation (Shi and Massague, 2003). Furthermore, there are substantial differences between activin A and activin C in residues important for type I receptor binding (Thompson et al., 2004). Finally, Wada et al. report activin C to act independently of activin A signaling in AML12 cells expressing a dominant-negativ type II activin receptor (Wada et al., 2004b). Taken together with our findings of activin C not to transduce signals through Smad2/3 we suggest other receptors to be involved in activin C signaling.

Besides the canonical Smad pathway TGFβ utilizes a multitude of intracellular signaling pathways to regulate its wide array of cellular functions. These include MAP Kinases like ERK, JNK and p38 (Derynck and Zhang, 2003; Itoh et al., 2000). Evidence of a cross talk respectively an alternative in activin signaling is found in our data with the activation of ERK by activin C. Interestingly, only PHH and HepG2, which do not show Smad phosphorylation in the basal state, utilize ERK. Data were supported by transactivation reporter assay. After activation of the ELK1 construct by cotransfection of ERK, activin C enhanced reporter activity in HepG2 but decreased it in Hep3b. Activation of ERK via TGFβ is known to involve phosphorylation of tyrosine residues on both type I and type II receptors and/or on Shc. The phosphorylated tyrosines are capable of recruiting Grb/Sos which then activate Ras, Raf and their downstream MAPK cascades to finally activate ERK (Lee et al., 2007). Besides, ERK can interfere with the Smad signaling cascade by regulating receptor activated R-Smad

activities through their phosphorylation (Kretzschmar et al., 1997; Kretzschmar et al., 1999; Matsuura et al., 2005). Our results indicate, that activin C does not necessarily utilize the canonical Smad pathway in liver derived cells. For the first time we show that the ERK pathway is activated by activin C only in cells with unphosphorylated Smad basal level.

Erk is widely known as target for cytokines rather promoting survival (Roux and Blenis, 2004) by inactivation of proapoptotic protein Bad or upregulation of antiapoptotic Bcl-2, Bcl-xL and Bcl-1 proteins via phosphorylation cytoplasmatic p90 ribosomal protein S6 kinase (RSK). (Sebolt-Leopold and English, 2006). In addition, ERK activity can suppress Fas mediated apoptosis by inhibiting the formation of the death-inducing signaling complex (DISC) (Holmstrom et al., 2000). However, there is evidence for its involvement in apotosis too depending on cell type and conditions. Inhibition of ERK by MEK inhibitors rescues several cell types from H_2O_2 or cisplatin induced cell death (Kim et al., 2005; Lesuisse and Martin, 2002; Nowak, 2002; Pavlovic et al., 2000; Wang et al., 2000). ERK activation is also associated with cell death induced by reactive oxygen species (ROS) or depravation of survival factors (Ramachandiran et al., 2002; Sinha et al., 2004; Tikoo et al., 2001). Finally, it is involved in doxorubicin induced apoptosis in human hepatoma cell lines. In our hands overexpression of ERK in HepG2 cells caused an increase of PI positive cells. As the phosphorylation deficient mutant did not have any effect on apoptosis, rise of PI positive cells is not an effect of overexpression but is due to activation of ERK mediated signaling pathways. Furthermore, treatment of ERK transfected HepG2 with the MEK1 inhibitor U0126 resulted in reduction of the ERK caused apoptosis. In fact numbers of PI positive cells were approximately the same as in untreated cells (data not shown). In Hep3b however, ERK had no effect on apoptosis, whereas the mutant caused an induction of PI positive cells. This may be due to blocking of endogenous signaling pathways in cells expressing much of the phosphorylation deficient mutant. Inhibition of endogenous ERK by U0126 caused a slight induction of apoptosis (data not shown).

By testing the effect of activins on viability we found that activin A acts in a viability inducing way in both HepG2 and Hep3b. These results are rather uncommon, as it is quiet confirmed in the literature that activin A acts as an apoptosis inducer in hepatoma cells (Chen et al., 2000; Schwall et al., 1993). However, Mashima et al. point out, that hepatoma cell lines are altered concerning their activin system and that effect of activin A are very much dependent on cell density (Mashima et al., 1995). Furthermore, many different conditions can increase or decrease metabolic activity. Changes in metabolic activity can give large changes in MTT results while the number of viable cells is constant. Hardly anything is known about the properties of activin B in the liver. Whereas activin A, C and TGFß had approximately the same effect on viability in HepG2 and in Hep3b, activin B had opposing effects. It caused an increase of viability in HepG2 cells but decreased viability in Hep3b. Concerning Hep3b data

are supported by reports showing transfection of this cell line with receptor Alk7, which conveys the response to activin AB and B (Tsuchida et al., 2004), to result in apoptosis (Kim et al., 2004a). It is very likely that not only the activin system is deranged in these cells but also the expression of specific receptors determining responsiveness to added factors. Data concerning activin C and TGFβ suggest a viability reducing function in both hepatomaderived cell lines, whereas effects are only significant in Hep3b.

TGFß is reported to cause apoptosis amongst others via activation of the p38 MAPK. It is for example activated after TGFβ treatment in vascular endothelial cells and in FaO hepatoma cells, whereas alteration of the two other MAPK signaling pathways JNK and ERK can modify the activation suggesting a crosstalk among the MAPK pathways. p38 activation promotes cytochrome c release from mitochondria in these cells (Hyman et al., 2002; Kim et al., 2004b; Park et al., 2002). In other cells however, p38 is the only MAP Kinase mediating the apoptotic effect and that in a Smad-independent manner (Yu et al., 2002). The cooperation between p38 and Smad signaling is important in again other cell systems to fully induce apoptosis. Smad 7 for example is crucial to provide structural support for the ALK receptor mediated activation of p38 (Edlund et al., 2003). TAK1 is induced in a Smad independent manner to induce rapid and transient p38 activation, whereas the delayed p38 activation requires Smad signaling (Dai et al., 2003; Takekawa et al., 2002; Yoo et al., 2003). Reduced viability in Hep3b induced by activin B, activin C and TGFß treatment was accompanied by strong p38 phosphorylation, whereas in HepG2 the phosphorylation of p38 together with prominent Smad activation induced by activin A and B caused an increase of viability.

Data gained from the viability assay are supported by the propidiumiodide flow cytometric analysis we performed. In HepG2 activin C and TGFβ increased the number of PI positive cells, whereas serum deprivation in Hep3b caused so much apoptosis that the already only minor effects seen in the viability assay were almost totally diminished under these conditions. Only the survival reducing effect of activin B is evident in the increased number of PI positive cells as well. The tendency seen in the viability assay and in PI FACS is the same, thus the two methods support each other even if the expressiveness of one alone is not that high. We also want to mention, that all activins cause a rise of PI positive cells in PHH whereas activin A and C excite the most effect (data not shown). All in all, we show for the first time that the ability of activin C to induce apoptosis gets lost during dedifferentiation of hepatoma cells. In the normal liver TGFβ exerts its effects by limiting the growth of hepatocytes in response to injuries by inhibiting DNA synthesis, blocking cell cycle progression and inducing apoptosis (Fabregat et al., 2007). Nevertheless, TGFβ is overexpressed during hepatocarcinogenesis and is then assigned with a tumor-promoting role rather than a tumor suppressive potential (Rossmanith and Schulte-Hermann, 2001). By

utilizing primary human hepatocytes and the relevant cell model systems for hepatocellular carcinomas HepG2 and Hep3b (Slany et al., 2009) we suggest a similar modulating role regarding apoptosis for activin C too. This might also explain the so far rather conflicting data regarding apoptosis and activin C. The studies worked with different cell systems, different methods of activin application and different end points all influencing the action of activin C and thus the outcome (Chabicovsky et al., 2003; Mellor et al., 2003; Vejda et al., 2003; Wada et al., 2004b).

Furthermore, our data offer correlative result: activin C induces ERK phosphorylation and induces apoptosis in HepG2, and transfection of the respective cell line with ERK results in increased apoptosis as well. Thus, we suggest activin C to induce apoptosis by phosphorylation of ERK. ERK as intracellular mediator of activin C induced apoptosis provides a potential cross-talk between activin C signaling and regulation of apoptosis.

ERK is known to regulate the apoptotic pathway on different levels (Zhuang and Schnellmann, 2006). Its activation is for example required for mitochondrial membrane depolarization, cytochrome c release and subsequent activation of Caspase 3 in HeLa cells and in renal cell lines (Kim et al., 2005; Wang et al., 2000). In our hands, serum deprivation significantly elevates Caspase 3 activity in both cell lines. The addition of the activins however, did not further enhance Caspase activity, but rather decreased it, indicating Caspase 3 not to be involved in activin induced apoptosis. This is in accordance with Pandey et al. who report Caspase 3 not to be involved in apoptosis of 5123tc rat hepatoma cells and postulate that the requirement for Caspase 3 mediated steps is cell type and condition specific (Pandey et al., 2000). Thus, other effector Caspases like Caspase 6 or 7, whichs roles are less clear, or a Caspase independent mechanism may conduct apoptosis. In the human hepatoma cell line Huh-7 for example, TGFβ increased Caspase 8 activity, followed by activation of Caspase 9 and 3 (Shima et al., 1999). Kim et al. show activation of Caspase 9 in ALK7 overexpressing FaO and Hep3b cells, thus suggesting a cross-talk with the cellular stress pathway. They also suggest a death ligand independent response because Caspase 8 was not activated in ALK7 induced apoptosis (Kim et al., 2004a). However, treatment with recombinant activin B, like it is the case in our experiments, might not be sufficient to elicit the same effects.

There are data suggesting ERK to be able to regulate apoptosis activating Caspase 8 (Cagnol et al., 2006). Caspase 8 in turn is able to activate proteins from the Bcl-2 familiy such as Bid, which catalyzes mitochondrial outer membrane permeabilization (MOMP). MOMP often defines the point of no return and not only results in release of cytochrome c but also in release of other mitochondrial constituents. This is for example the apoptosis inducing factor (AIF), which's release can be triggered by the lysosomal protease cathepsin too

(Bidere et al., 2003; Cande et al., 2004). Another example is the serine protease HtrA2/Omi contributing to effector Caspase-independent apoptosis (Kroemer and Martin, 2005).

Taken together the complexity of cell death regulation is conditioned by the functional redundancy within the family of Caspases, but also by the adaptability of the apoptotic pathway which, once initiated, goes to completion by accessing other available cellular players. Furthermore, cross-talks with signaling pathways contribute to diversity of cell death execution. Regarding the TGFβ superfamily members activins elucidation of the exact receptors and signaling mechanisms will contribute to better understanding of biological functions like apoptosis, whereas it will be quite interesting to distinguish different modes of action for the respective activins.

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3. Additional Data

Activin Cs role in viability, apoptosis, migration and inflammation in different cell systems.

Abstract

TGFβ family members, including activins, are essential regulators of diverse physiological mechanisms. They contribute to organogenesis and formation of basic structures of solid organs during development and are widely expressed during rodent embryonic development. Mice deficient for InhβA or/and InhβB develop to term, though showing defects concerning whiskers, lower incisors or eyelid closure. Mice deficient for the highly liver specific InhβC or/and InhβE are viable without obvious abnormalities. Activin A, like TGFβ, has been related to apoptosis and is known to inhibit DNA synthesis and proliferation. Data on activin Cs effect on cell growth and apoptosis are rather conflicting, as in different cell lines and different settings activin C overexpression or treatment with recombinant activin C resulted in increased growth and cell division or in induction of apoptosis. Apoptosis is an essential process for normal development, tissue homeostasis, host defense, suppression of oncogenes and therefore cancerogenisity. Two other biological mechanisms also important in cancerogenicity are motility phenomena and inflammation. Though TGFβ is well known to contribute to epithelial to mesenchymal transition and thus motility, nothing is known so far about activins and metastasis. Concerning inflammation the involvement of activins has become topical just recently. Activin A for example is significantly elevated in the circulation following induction of an inflammatory response with LPS. It also exerts anti-inflammatory effects in HepG2 by inhibiting the induction of acute phase protein synthesis by IL6. Similarily activin C decreases secretion of several acute phase proteins like T-kininogen.
The following work tries to shed light on some aspects of TGFβ family members' fields of action. It is also an approach for finding a biological role of activin C, for which, in contrast to activin A, hardly anything is known so far about its impact on physiological mechanisms.

Introduction

The TGFβ family as a whole, as well as the activins, is an essential regulator in diverse physiological mechanisms. It affects embryonic development, inflammation, cell proliferation, apoptosis and thus metastasis and cancerogenisity. In the following work, we tried to shed light on some aspects of these interwoven fields, by utilizing different cell systems. It is also an approach for finding a biological role of activin C, for which, in contrast to activin A, hardly anything is known so far about its impact on physiological mechanisms.

Development

Activins contribute to organogenesis and formation of basic structures of solid organs during development (Asashima et al., 1999; Ball and Risbridger, 2001). They are widely expressed during rodent embryonic development, though at different time points and in different tissues. InhβA and InhβB subunits are expressed in the zygote, after implantation however, they are only expressed in the maternally derived deciduum (Albano et al., 1993). On day 10.5 expression of the InhβA subunit mRNA arises in mesenchymal cells of the developing face, whiskers, hair follicles, heart and digestive tract, whereas the βB subunit mRNA is expressed in the brain, spinal cord and the epithelium of the stomach and the esophagus (Albano et al., 1994; Albano et al., 1993; Feijen et al., 1994; Lu et al., 1993; Manova et al., 1992; Smith et al., 1990; Thomsen et al., 1990). Mice deficient for activin InhβA or InhβB or both develop to term. InhβA deficient mice lack whiskers and lower incisors, have defects in their secondary palates and die within 24h after birth. InhβB mutant animals suffer from eye lesions and reproductive defects. Mice lacking both subunits showed additive combinations of the single mutant phenotype, indicating the two activins do not compensate for each other during development. In contrast to Xenopus laevis, were InhβA is known to be involved in mesoderm formation, zygotic expression of activins is not essential for mesoderm induction in mice (Matzuk, 1995; Matzuk et al., 1995; Thomsen et al., 1990; Vassalli et al., 1994).

The highly liver specific InhβC subunit expression was detectable at day 11.5 and 14.5 depending on detection method, reaching its maximum at birth, whereas InhβE subunit expression was not detectable until day 17.5, and reached its maximum a few weeks after birth (Lau et al., 2000). Mice in which either one InhβC or activin InhβE or both subunits had been knocked out were viable without obvious abnormalities in liver size and function, suggesting that they are not necessary for mouse embryonic development.

Based on the knowledge of activin expression during development, we hypothesized that cells are reactive for the activins not until the respective subunits are expressed in the organism. As the embryonic development is affected by proliferation and apoptosis, we

performed viability assays with REC cells. These cultures are prepared from 13.5, 14.5 and 15.5 day-old Fischer rat embryos, thus addressing different phases of activin subunit expression.

Apoptosis

TGFβ appears to be central in all the multiple events of proliferation and apoptosis. Whereas it is growth stimulatory for cells of mesenchymal origin, it works growth inhibitory on cells of epithelial origin. In the liver it is one of the few identified negative regulators of liver cell mass, acting by induction of apoptosis (Oberhammer et al., 1991; Whitman, 1998).

Activin A, like TGFβ, has been shown to inhibit DNA synthesis and proliferation and to induce apoptosis in hepatocytes. It has also been related to apoptosis and induction of cell cycle arrest in hepatoma cell lines (Hully et al., 1994; Schwall et al., 1993; Yasuda et al., 1993). Furthermore, administration of the activin A antagonist follistatin in vivo by intraportal infusion or adenovirus-mediated overexpression caused liver mass and DNA content growth by blocking the effect of activin A (Kogure et al., 2000). Activin B, unlike activin A, did not inhibit DNA synthesis in primary cultured rat hepatocytes (Niimi et al., 2002). Nevertheless, rat and human hepatoma cell lines infected with ALK7, which is supposed to be the preferred type I receptor of activin B, undergo apoptosis (Kim et al., 2004a). Data on activin Cs effect on cell growth, DNA-synthesis and apoptosis are rather conflicting. Thus, in different cell lines and different settings overexpression of activin βC subunit or treatment with recombinant activin C resulted in increased growth and cell division or in induction of apoptosis (Vejda et al., 2003; Wada et al., 2004). Mellor et al. however, did not find any effect of activin C on apoptosis and cell growth at all (Mellor et al., 2000). More consistent data are available on activin E, whereas it was associated with growth inhibition and apoptosis induction in hepatoma cells as well as in immortalized mouse hepatocytes (Oberhammer et al., 1996; Risbridger and Cancilla, 2000).

We studied the effect of the TGFβ subfamily on apoptosis using the embryonic rat cell line 423, a well established apoptosis cell model.

Signaling

Activins like other members of TGFβ family exert their biological actions through heterodimeric complexes of type I and type II serine/threonine kinase receptors (Attisano et al., 1996; Derynck and Feng, 1997; Mathews, 1994). Activin binding to type II receptor recruits the type I receptor, which is activated upon phosphorylation and passes the signal to downstream proteins (Attisano et al., 1996; Zimmerman and Mathews, 1996). Two type II receptors for activin A (ActRII and ActRI have been identified (Attisano et al., 1992; Carcamo et al., 1994; Mathews and Vale, 1991; Tsuchida et al., 2004). Its main type I receptor is

ALK4, also known as ActRIB, whereas activin B and AB prefer Alk7 (Tsuchida et al., 2004). Signals from TGFβ and activin A receptors I are transmitted to the transcription factors Smad2 and Smad3, which are recruited to the receptor complex and activated by phosphorylation (Siegel and Massague, 2003). The phosphorylated R-Smad and the Co-Smad 4 form complexes that translocate to the nucleus to activate the transcription of target genes. About receptors and downstream signaling for activins containing βC or βE subunits hardly anything is known so far, as there are only two reports concerning this topic (Gold et al., 2009; Mellor et al., 2003). They show that activin C does not signal through Smad2/3 in human prostate tumor cell lines, respectively pituitary cell lines.

As shown before signaling induced by activin C differs between the hepatoma cell line HepG2, which still exhibits many features characteristics for primary hepatocytes, and Hep3b, which already express some mesenchymal proteins. Whereas signaling does not work via Smad2/3 phosphorylation in both of the cell lines, activin C induces ERK phosphorylation only in HepG2. We were interested if there are similarities respectively differences compared to signaling mechanisms of fibroblast cells. Hence we utilized the rat fibroblast 423-cells, which are well known apoptosis models.

Inflammation

One property of the activin subfamily that has become topical just recently is their involvement in inflammation. Philips et al. first showed elevated circulating follistatin levels after generation of inflammatory responses in in vivo sheep models (Phillips et al., 1998; Phillips et al., 1996). Also activin A is significantly elevated in the circulation following induction of an inflammatory response with LPS (Jones et al., 2000). It even gets released earlier than tumor necrosis factor alpha (TNFα), which is one of the earliest cytokines released in inflammation, as well as before any elevation of IL6 can be measured (Jones et al., 2004). Thus, activin A seems to be a very early and critical component of acute inflammation.

The organism responses to inflammation with well coordinated changes in systemic, metabolic, and local functions all together known as acute phase response. One of the most important features of the acute phase response is the rapid decrease or increase of several plasma proteins, the so called acute phase proteins (APP). All of them are synthesized in the liver and may be regulated by IL1 or IL6 pathways (Moshage, 1997). TNFα for example is released by kupffer cells upon exposure to lipopolysaccharides (LPS) - glycolipids found on the membrane of all gram-negative bacteria – which are the most important bacterial endotoxins. TNFα in turn works on hepatocytes and endothelial cells, triggering a cascade of other cytokines (Su, 2002). C-reactive protein (CRP) is induced by IL6 in hepatocytes, and plasma levels of human CRP may rise rapidly and markedly after an acute inflammatory

stimulus. In contrast, plasma levels of rodent CRP rarely increase following inflammatory stimuli, making it a rather improper end point when studying inflammatory responses in rodent (Black et al., 2004). Inflammatory responses mediated by IL6 also stimulates T-kininogen production in hepatocytes (Anderson and Lingrel, 1990). T-kininogen belongs to the kallikrein-kinin system which participates in physiological and pathological processes like inflammation.

Activin A itself exerts anti-inflammatory effects in the hepatoma cell line HepG2 by inhibiting the induction of APP synthesis by IL6 (Werner and Alzheimer, 2006). A similar action has been found for activin C. Proteome analysis of primary rat hepatocytes treated with inducers of APP followed by human recombinant activin C revealed alterations in synthesis of numerous proteins. These included decreases in secreted APP T-kininogen, fibrinogen β- and γ-chain and fetuin A while synthesis of transthyretin, albumin and apolipoprotein A-1 was increased (Erlach et al, in preparation).

In this report, we show that activin C decreases basal as well as IL6 induced T-kininogen mRNA expression in PRH. Furthermore, we examined activin Cs effect on TNFα secretion in rat Kupffer cells and performed a CRP ELISA with the supernatant of activin treated primary human hepatocytes, to assess their effect on secretion of a human APP.

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Migration/EMT

Growth factor induced motility phenomena, respectively epithelial-mesenchymal transition (EMT), are essential for embryonic growth and development, wound healing, angiogenesis, metastasis of neoplastic tumors and regeneration of tissues. In all of these processes TGFβ family members are known to play important roles, and many of them are crucial in the liver.

Activin A has been linked to liver fibrosis, as several investigations have found elevated levels of it in fibrotic and cirrhotic rat livers as well as circulating activin A in patients suffering cirrhotic viral hepatitis or alcohol induced cirrhosis (Gold et al., 2003; Huang et al., 2001; Patella et al., 2001; Sugiyama et al., 1998; Yuen et al., 2002). Furthermore, it has been implicated in neoangiogenesis via stimulation of VEGF expression in human hepatoma cells (Wagner et al., 2004). In regenerating livers after partial hepatectomy InhβA expression has been shown to drop to less than 50% of control values, allowing hepatocytes to replicate, and increased again to three times above control after 168h when liver mass was fully restored (Gold et al., 2005). InhβB mRNA expression was upregulated in stellate cells of CCl_4 treated rat livers, and in livers exposed to the peroxisome proliferator di-n-butyl phthalate (De Bleser et al., 1997; Kobayashi et al., 2002). The same treatments also resulted in increase of InhβC expression (Gold et al., 2003; Kobayashi et al., 2002). Furthermore InhβC contributes to liver regeneration, as its adenovirus-mediated expression accelerated regeneration after partial hepatectomy in rats (Wada et al., 2005a).

Beside other growth factors, epidermal growth factor (EGF) is a potent stimulator of EMT in several cell types in a direct manner, as it is involved in E-cadherin expression and collagen remodeling, but also indirectly by conferring apoptotic resistance and mitogenic potential to cells undergoing EMT. Both events are associated with increases in the invasive potential of cancer cells and have been identified as key steps in EMT (Hoschuetzky et al., 1994; Hugo et al., 2007; Klymkowsky, 2005; Nelson and Nusse, 2004).

Whereas TGFβ is well known to contribute to EMT and thus to motility of cells, hardly anything is known about activins and metastasis so far. Hence, we were interested in whether activins may contribute to motility of hepatoma cells, and if they are able to influence EGF induced motility.

Material and Methods

Cell culture

Primary rat hepatocytes (PRH) and Kupffer cells (PRK) were kindly isolated by Sandra Sagmeister (Cancer Research Institute, Vienna, Austria) as described previously (Parzefall et al., 2001). Hepatocytes were seeded on collagen-coated petri dishes, with the collagen prepared according to the method of Ehrmann and Gey (1956), and kept under serum free conditions in Williams Medium E (Gibco). Treatment of the PRH was performed 4 hours after plating. Kupffer cells required no coating and were kept in RPMI 1640 (Gibco) with 10% FCS (PAA Laboratories). Treatment of the PRK was accomplished 2 hours after plating.

Primary human hepatocytes (PHH) were allocated by courtesy of Thomas Weiss (University of Regensburg) as described previously (Weiss et al., 2002; Weiss et al., 2003). In brief, tissue samples from liver resection were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research), with the informed patient's consent approved by the local ethical committee of the University of Regensburg. Cells were cultivated in serumfree Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 7ng/ml glucagon, 0.5U/ml insulin, 8µg/ml hydrocortison, 100 U/ml penicillin/streptomycin and 2nM L-glutamine. Treatment was performed 72 hours after plating.

Human hepatoma cell lines HepG2 and Hep3 as well as the rat fibroblast cell line FR3T3 from ATCC were propagated in RPMI 1640 (Gibco) supplemented with penicillin/streptomycin (100 U/ml; Sigma) and 10% FCS.

Activin C expressing CHO cells as well as mock transfected CHO cells were generated (Vejda et al., 2003) and kindly provided by Jens Pohl (Biopharm GmbH, Heidelberg, Germany). Like the embryonic rat cell line 423, immortalized by Human Papilloma Virus type 11 DNA (Cerni et al., 1990), and REC cultures prepared from 13.5, 14.5 and 15.5 day-old Fischer rat embryos (Cerni et al., 1990), they were routinely cultivated in DMEM (Firma), supplemented with penicillin/streptomycin (100 U/ml; Sigma) and 10% FCS.

All of the culture incubations were carried out at 37°C in humidified atmosphere containing 5% CO_2 for cells cultivated in RPMI/Williams Medium E and 7.5% CO_2 for cells propagated in DMEM.

MTT-Assay

To investigate cell viability we used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay based on mitochondria metabolic activity. It was applied to different cell lines, namely RECs and 423-cells. A total of $3x10^3$ RECs respectively $30x10^3$ 423-cells were added to each well of a 96-well microtiter plate and allowed to attach overnight. They were then treated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ (R&D Systems) in serumfree RPMI/DMEM for 48 hours. 2.5 ng/ml FGF2 (BioVision) served as positive control in 423-cells. MTT (Fluka) was dissolved to a concentration of 5 mg/ml in PBS and filtered for sterility. Cells were then washed once with PBS and 100µl DMSO (Sigma) were added to each well to lyse the cells. Absorbance was immediately measured at 550nm using an automatic micro-plate reader (Synergy HT).

In situ detection of apoptotic cells

Briefly, 423 cells cultured and treated with 10ng/ml of the respective TGFβ family member for 6 hours on 24well plates were fixed with 4% formaldehyde in PBS (Sigma) at room temperature for 20min and washed with PBS once. Nucleic staining was performed using a 1:1000 dilution of a 5mg/ml DAPI stock solution (4',6-Diamidin-2'-phenylindol- dihydrochlorid, Roche Diagnostic). After incubation for 5min, cells were washed with PBS and stored covered with PBS up to several days. Fields in each of the wells were randomly chosen before visual inspection, and at least 300cells/well were counted on a Nikon Eclipse TE 300 microscope. Apoptotic cells were expressed as percentage of the total cell number.

Analysis of apoptosis by flow cytometry

423-cells were seeded and grown to confluence in six-well plates. Three wells each were treated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in serumfree medium and harvested 24 hours later. Three different detection methods were used, namely JC1 for detection of mitochondrial depolarization occurring during the early stage of apoptosis, propidium iodide for detection of late apoptosis, and a Caspase assay to measure cleaved Caspases as indicator for apoptosis.

For JC1 and PJ detection method cells were harvested through trypsinization, floating and attached cells were combined and washed twice with cold PBS. The cells were then centrifuged at 3000 r/min for 5min and the supernatant was discarded. The pellet was resuspended and incubated with 5 µM JC1 (Invitrogen), respectively 10µl of propidium iodide (30µg/ml, Invitrogen) for 15 min at room temperature in the dark, and then analyzed in a Becton Dickinson FACSCalibur system using CELLQuest software. The Caspase assay was performed according to manufacturer's introduction (BioVision).

Western blotting

Cells were solubilized in a lysis buffer containing 50 mM TRis·Cl pH 7.4, 500mM NaCl, 1% NP-400, 5% Na-Deoxycholat, 0.1% SDS0, 0.5% NaN_3, 100mM Na_3VO_4 and the protease inhibitor complete (Roche Diagnostics). Proteins were separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfered to PVDF membranes (Amersham Biosciences). Blots were blocked with 10% low fat milk in PBS with 0.05% Tween 20. The following primary antibodies were used at a dilution of 1:1000: Smad2/3 (Cell Signaling Technology Inc.), ERK, p38, JNK plus the respective ones for the phosphorylated forms, and βActin (all from Sigma) as loading control. Additionally Bax and Caspase 3 (Santa Cruz) antibodies were used. Subsequently, corresponding secondary antibodies (Calbiochem) were applied, and visualization was performed with an enhanced chemiluminscense detection system (Thermo Fisher Scientific) according to the manufacturer's instructions. The blots were on the one hand exposed to X-ray film, on the other hand detection was performed using a Chemi Smart 5001 (Peqlab). Quantification was performed using Image Quant.

Luciferase assay

423-cells cultured in 24-well plates were transiently transfected with 1 µg of the indicated constructs using Fugene 6 (Roche) transfection reagent following manufacturer's protocol. To correct for transfection efficacy, 100ng Renilla luciferase (pRL-CMV, Promega) were cotransfected. pGL3 vector DNA was used to keep the total amount of DNA in all samples constant. On the next day cells were washed with PBS and treated for 6 hours with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in RPMI containing 2%FCS. Luciferase activities were quantified using Dual-Luciferase Assay (Promega) according to manufacturer's instructions. Emitted luminescence was measured using a Labsystems Luminoscan. Firefly luciferase activity was normalized for renilla luciferase activity.

Luciferase reporter gene constructs. For intracellular signaling of TGFβ, a cis-acting CAGA-luciferase construct, consisting of 12 Smad3/Smad4 binding sequences (CAGA boxes) and the luciferase-coding sequence, was used. The CAGA boxes confer TGFβ stimulation to a promoter reporter construct, whose activity depends on binding of activated Smad3/Smad4 transcription factor complexes (Dennler et al., 1998). Additionally, a 3TP-Luc plasmid, which besides the luciferase coding sequence consists of plasminogen activator inhibitor 1 (PAI-1) gene promoter containing three repeats of TGFβ–responsive element and Smad2/3, was used (Wrana et al., 1992). Plasmids were kindly provided by Wolfgang Mikulits (Cancer Research Institute, Vienna, Austria).

RNA isolation, RT-PCR and Real Time RT-PCR

Cells were cultured in 6-well plates and treated with 10ng/ml of the respective activin for 24 hours. They were then washed with ice cold PBS once and lysed directly in the culture dish by adding 300µl of Trizol reagent (Invitrogen) and scraping with a cell scraper. The lysate was treated with 0.5xVolume chloroform, shaken thoroughly and incubated at room temperature for 15 minutes. A centrifugation step was carried out for 15 minutes at 12000 g and 4°C. Then 7x volume isopropanol was added to the aqueous phase and incubated over night at -80°C. Centrifugation for 8 minutes at 12000 g and 4°C followed. The precipitated RNA was washed with 0.3 ml ethanol and again centrifuged for 8 min at 12000 g and 4°C. After carefully removing the ethanol, the RNA was dissolved in diethylpyrocarbonate treated water (Sigma) and the RNA concentration was determined from the optical density at the wavelength of 260 nm on GenQuant (Pharmacia). For optimal purity the ratio 260/280nm was framed between 1.7 and 2.0. An amount of 2µg total RNA was transcribed into cDNA applying random-dT primers for 1h at 42°C using MMLV RevertAid (Fermentas). 20µl cDNA were filled up to 100µl before usage.

Following T-kininogen primers were used for RT-PCR: 5' AGTTAGAAAGCGGCAACCAG 3' as forward primer and 5' TGTTGTGCATTCGCCAGTAG 3' as reversed primer. Genes of interest were amplified from cDNA samples by standard PCR cycles consisting of 30 seconds denaturation (95°C), 20 seconds annealing (62°C) and 20 seconds synthesis (72°C). Products were separated on 6% acrylamide gels using Vistra Green (GE Healthcare) for staining. A Fluor Imager 595 was used for quantification.

Two microliters cDNA were used as a template for each Real Time RT-PCR. Analysis was performed with TaqMan® Universal PCR Master Mix (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) running the following protocol: initial 50°C 2min and 95°C 10min, and 40 repeats of 95°C 15 sec, 60°C 1 min. Taqman assays for human C-reactive protein (CRP, Hs00357041_m1), and β2-microglobulin (B2M; Hs99999907_m1) were obtained from Applied Biosystems furthermore following Taqman assays for rat were used: βA (INHBA Rn00567500_m1), βC (INHBC Rn00575117_m1) and β2-microglobulin (B2M Rn00560865_m1). All samples were analyzed in duplicate in at least two independent Real Time RT-PCR runs.

ELISA

Primary rat Kupffer cells were treated with 10ng/ml of the respective recombinant human activins and different concentrations of LPS for 48 hours. TNFα levels in PRK supernatants were measured by the rat TNFα Module Set (Bender MedSystems) according to the manufacturer´s instructions. Human primary hepatocytes were treated with 10ng/ml of the respective recombinant human activins and IL6 for 48 hours. CRP levels were measured in

PHH supernatans by human C-reactive protein Instant Elisa (Bender MedSystems) according to the manufacturer´s instructions.

Transwell chemotaxis assay

To study the effect of activins on motility and mitogenesis Hep3b cells were seeded into the upper part of 8µm pore Transwell chambers at $3x10^4$ cells/ml. They were allowed to attach for several hours, were washed twice with PBS and new serum free RPMI was added. At this time the motility assay was initiated by transferring the chambers to a new 24 well plate containing the following factors: recombinant human activin A, B and C as well as TGFβ each at 10ng/ml, and 10ng/ml EGF (Sigma) as positive control. The assay was allowed to continue for 24 hours prior to termination. Cells were fixed by immersing the Transwell in 4% formaldehyde in PBS for 20 min. Cells were stained using a 1:1000 dilution of a 5mg/ml DAPI stock solution (4',6-Diamidin-2'-phenylindol- dihydrochlorid, Roche Diagnostic). Transwells were washed, and stationary cells were removed from the inside of it by gently rubbing with a cotton-tipped applicator. The filters were fixed on a slide and then photographed using a Nikon Eclipse TE 300 microscope equipped with a Nikon Digital Camera DS. Images where then analyzed using Image J freeware (*rsbweb.nih.gov/ij/*).

Scratch assay

The scratch assay is a common method to measure cell migration in vivo. Therefore Hep3b cells were allowed to grow till they formed a confluent monolayer. This monolayer was scratched using a pipette tip, introducing a cell free area. Cells were washed twice with PBS and further kept in serum free RPMI. 10ng/ml recombinant human activin A, B and C and TGFβ (R&D Systems) were added, 10ng/ml EGF (Sigma) served as positive control. The open gap was inspected microscopically over a time period of 24 hours, as the cells moved in and filled the damaged area. Pictures were taken on a Nikon Eclipse TE 300 microscope equipped with a Nikon Digital Camera DS and analyzed using MetaMorph 7.5 software.

Statistical analysis

The experiments were done in 2-fold and repeated at least three times. One sample t-test was applied to determine statistical differences using GraphPad Prism 5 package (San Diego). Values are expressed as mean ± SD, with $p<0.05$ considered significant.

Results

Activin C enhances viability in rat embryonic cells depending on their stage of development

Investigation of accessibility of cells for activins during embryonic development was performed regarding viability of cells prepared from 13.5, 14.5 and 15.5 day-old Fischer rat embryos. InhβA mRNA expression is reported to arise on day 10.5, expression of InhβC between day 11.5 and 14.5. In our hands, InhβA expression decreased to 25% comparing REC13.5 and REC15.5, whereas InhβC expression doubled (data not shown). In the MTT assay cells from 13.5 day old rat embryos respond to treatment with activin A with enhanced viability, whereas activin C hardly had any effect. Cells from day 14.5 reacted to activin A in a concentration dependent manner: concentrations under 10ng/ml enhanced viability, concentrations above 10ng/ml reduced viability. Activin C treatment again had only minor effects on viability, although there already was a slight positive effect detectable. When applied to cells from day 15.5. activin A had no or rather inhibiting effects on viability, whereas activin C enhanced viability. These data suggest, that activin A and C effect viability of rat embryonic cells during development depending on the day of growth.

Activin C act as survival factor in 423 rat fibroblasts

To determine the effect of activins on viability in 423-cells, respective cells kept in serum containing 2% FCS were incubated with 10ng/ml of recombinant activin A, activin B, activin C and TGFβ. FGF2, which has been reported to mediate cell survival in 423-cells, served as positive control (Schamberger et al., 2004). All tested factors could increase viability compared to serum reduction, whereas the activins led to a significant 50% increase compared to control. Addition of TGFβ resulted in a doubling of viability (Fig.2A). After weakening the cells not only by serum reduction but also by administering cisplatin, the effects of the above mentioned factors were differential according to concentration of cisplatin (Fig.2B). At relatively moderate cisplatin concentrations of 2.5mM all activins and TGFβ were able to save the 423-cells from cisplatin induced loss of viability. FGF2 promoted survival the most. However, at higher concentrations, the TGFβ family members rather enhanced loss of viability (Fig.2C).

To investigate the outcome of TGFβ family treatment on apoptosis, 423-cells were induced to undergo apoptosis by serum withdrawal, and treated with 10ng/ml of the respective factors for 6 hours. We found that serum starved 423-cells rounded up drastically, the same did activin A treated cells, whereas activin B treatment already reduced the rounding up to some extent. However, cells treated with activin C and TGFβ did show these apoptotic characteristics to a much lesser extent (Fig.3B). These findings were supported by counting

the nuclei containing condensated DNA: serum deprivation caused approximately 100% more apoptosis than cells kept in 10% FCS. Activin A and activin B enhanced survival by nearly 20%. Activin C and TGFβ even caused a rise of survival of about 50%, and thus turned out to be the most potent survival factors of all the applied TGFβ superfamily members in 423-cells. To further analyze the involvement of activins in apoptosis respectively survival, we performed Westernblot analyses focusing on the proapoptic protein Bax, and the apoptosis executer Caspase 3. Detection of Bax protein supported conclusions achieved by microscopical analysis of apoptotic nuclei: Bax expression induced by serum withdrawal was slightly reduced by treatment with activin A and B, but clearly reduced after addition of activin C and TGFβ (Fig.3C). Detection of Caspase 3 however, did not show satisfying results, that's why we performed a flow cytometric Caspase assay. Activin treatment resulted in reduced Caspase activity compared to control cells. However, the most reducing effect had TGFβ (Fig.4A). These data suggest the activin subfamily to be involved in survival of 423 rat fibroblastoid cell, and activin C turned out to be the most potent survival factor within this group.

As apoptosis is characterized by loss of mitochondrial membrane potential and cytochrome c release, we further examined changes in mitochondrial membrane potential after activin treatment by flow cytometric analysis of JC-1 complexes. The percentage of 423-cells with low mitochondrial membrane potential decreased slightly after activin A and B treatment. Activin C did not have any effect on the mitochondrial membrane potential compared to control cells, whereas TGFβ even reduced the 423-cell number with low mitochondrial membrane potential to less than 50% (Fig.4B). However, loss of mitochondrial potential is an early event in apoptosis. We additionally performed a flow cytometric analysis using propidiumiodide, thus addressing rather late events of apoptosis. With this method activin C even had a proapototic effect on the serum starved 423-cells (Fig.4C).

Activin C does not primarily signal via Smad2/3 in rat fibroblasts

As shown signaling induced by activin C differs between the hepatoma cell line HepG2, which still exhibits many features characteristics for primary hepatocytes, and Hep3b, which already express some mesenchymal proteins. Whereas signaling does not act via Smad2/3 phosphorylation in both cell lines, activin C induces ERK phosphorylation only in HepG2. To determine the signaling mechanisms in fibroblastoid 423-cells, respective serum starved cells were treated with 10ng/ml activins and TGFβ for 30min. Total lysates were analyzed by Western blotting using a specific antibody to phosphorylated, thus activated Smad. The membrane was reprobed with a specific Smad antibody and an antibody to βActin, to show equal loading of the proteins. Additionally determination was performed by a transcriptional activation assay, whereas cells were exposed to the activins in medium containing 2% FCS

for 6 hours. The reporter construct used in this study is CAGA, which contains a luciferase gene under the control of a synthetic promoter whose activity depends on binding of activated Smad3/4. Activin A, activin B and TGFβ clearly induced activation of Smad on protein level, whereas treatment with activin C reduced the basal level of Smad phosphorylation (Fig.5B). In contrast, CAGA-luciferase activity was slightly induced by all factors used (Fig.5A). Usage of a second luciferase reporter construct, namely 3TP-Luc, proved these findings (data not shown). Furthermore, we explored the ability of activins to activate MAPK/SAPK signaling cascade. Certainly, neither ERK, nor JNK and p38 were activated upon activin treatment (data not shown). These results in 423-cells indicate that activin C, in contrast to activin A and B, does not signal via Smad2/3 in fibroblast cells. Additionally there is no activation of Smad independent pathways, raising the question of other signaling mechanism. Again, we show that serum conditions are an important factor when studying functions of activins in vitro, as their effects can be weakened or inhibited by serum added to the medium.

Modulation of inflammatory responses in rat and human primary liver cells

In order to study possible effects of the activin subfamily on induction of acute phase proteins in primary rat hepatocytes we first performed a PCR using primers for T-kininogen, a major acute phase protein in rat hepatocytes. Hence activin A and activin C reduced the basal T-kininogen mRNA expression, while activin B increased it. Furthermore, activin A and activin C decreased IL6 induced T-kininogen mRNA expression (Fig.6), which is according to findings by Erlach et al. They showed activin C to decrease IL6 induced T-kininogen secretion on protein level (Erlach et al., in preparation).

Kupffer cells, liver specific macrophages, contribute to the acute inflammatory response by secretion of TNFα. We performed a TNFα Elisa with supernatant of respective LPS and activin stimulated rat cells. Activin A, B and C did not alter basal TNFα secretion (Fig.7A). Interestingly, when TNFα secretion was induced by LPS activin A and activin B reduced the TNFα secretion, hence the inflammatory response, whereas activin C even enhanced the release of TNFα. This is what happened at relatively low LPS concentrations of 10ng/ml (Fig.7B). When the concentration was elevated up to 100ng/ml activin A and B were no longer able to reduce the inflammatory response. Activin C however retained its stimulatory effect on TNFα release (Fig.7C).

To address inflammation in humans, supernatant of activin treated primary human hepatocytes gained from four different patients was analyzed by CRP ELISA and Real Time RT-PCR. It has to be noted, that in contrast to primary hepatocytes gained from inbred rat strains, primary human hepatocytes are derived from individuals with divert genetical and health backgrounds. Thus, all samples analyzed revealed different outcomes (Fig.8A). In

patient 1 activin A had no effect, activin B caused a significant 2-fold increase of CRP secretion compared to control, and activin C resulted in a 4-times significantly elevated CRP secretion. In the second patient only activin B remained consistent with the results seen in patient 1 – it caused a significant 2-fold increase of CRP secretion compared to control. Activin A however, 6-times significantly magnified the CRP secretion, whereas activin C led to a decrease of CRP release. In the following two patients activin A caused a significant 2-fold increase of CRP secretion and activin B and C once slightly increased and once slightly degreased CRP secretion. Also induction with IL6, which is supposed to activate CRP secretion, did generate diverse data (Fig.8B). Only the second patient turned out to be accessible to IL6, and CRP secretion increased about 6 times. None of the other patients showed any effect to IL6 treatment. In patient 1, in which activin C treatment led to a 4-fold increase of CRP release, combined IL6 and activin C treatment did not show any effect on CRP secretion. Patient 2 supernatant, the only one responsive for IL6 stimulation, showed a slight decrease of CRP secretion when IL6 and activin C were administered together compared to treatment with IL6 alone. Patient 3, not responding to IL6 alone, showed an increase of CRP release with combined treatment. CRP levels in patient 4 supernatant however where halved no matter which combination of treatment was used. All in all CRP induction through activins varies in the individual organisms, which may be due to inflammatory events already taken place in the body. These preceding actions may work as trigger for the activins, determining their role as modulators in inflammation.

To specify the mechanisms concerning CRP induction through the activins, we exemplary picked patient 1, which did not respond to IL6 treatment, and patient 2, whose CRP level in the supernatant was elevated upon IL6 administration, for further analysis using Real Time RT-PCR (Fig.8C)

. Hence activin C alone could not elevate CRP mRNA in patient 1, though it increased protein level. Contrariwise the effect of IL6: while it did not cause induction of the CRP protein, the mRNA was elevated up to 100-fold. Combination of activin C and IL6 induced CRP mRNA even about 150-fold, whereas no such effect was seen on protein level. This reversed effect also turned up in patient 2, as activin C decreased CRP protein secretion but increased its mRNA. Effects of IL6 administration and of combined treatment however, were the same regarding protein and mRNA level as in both cases IL6 induced CRP level and activin C reduced this IL6 triggered CRP level. Taken together, there is a difference between activin C and IL6 induced CRP mRNA expression and CRP protein secretion. Thus, the administered factors operate at least at two different levels namely at the transcriptional and the translational one. As there is hardly any CRP protein detectable intracellular, they might also influence secretion.

Reduction of growth factor induced motility by activins in hepatoma cells

To study the effect of activins on motility and mitogenesis, Hep3b were examined by using a transwell chamber motility assay in conjunction with parallel scratch assays. 24 hours serum free conditions were used throughout the assays. As expected, EGF clearly induced motility of the cells compared to control in the transwell chemotaxis assay. The TGFβ family members however reduced motility to approximately 50% of control. When administered simultaneously with EGF they were even able to reduce the EGF induced motility effect, whereas activin C and TGFβ had the most reducing effect (Fig.9A). When examining the scratch assay, the motility reducing ability of the TGFβ family members was confirmed. Again, all of them reduced adhesion of the scratch to less than 50% of control (Fig.9B). We confirmed these data by testing another cell line, namely FR3T3. While Hep3b exhibit epithelial but also mesenchymal features, FR3T3 are fibroblasts. Still, activin C is able to inhibit closure of the scratch compared to control. As a second factor we used supernatant from activin C overexpressing CHO cells, as well as supernatant of mock transfected CHO cells not able to produce activin C. The effect of the supernatant containing activin C is indicated relative to the effect of the control supernatant, showing the same reducing ability as recombinant human activin C (Fig.9C).

These experiments demonstrate that activins act in a motility inhibiting way in hepatoma and fibroblastoid cells, and additionally are able to reduce EGF induced motility.

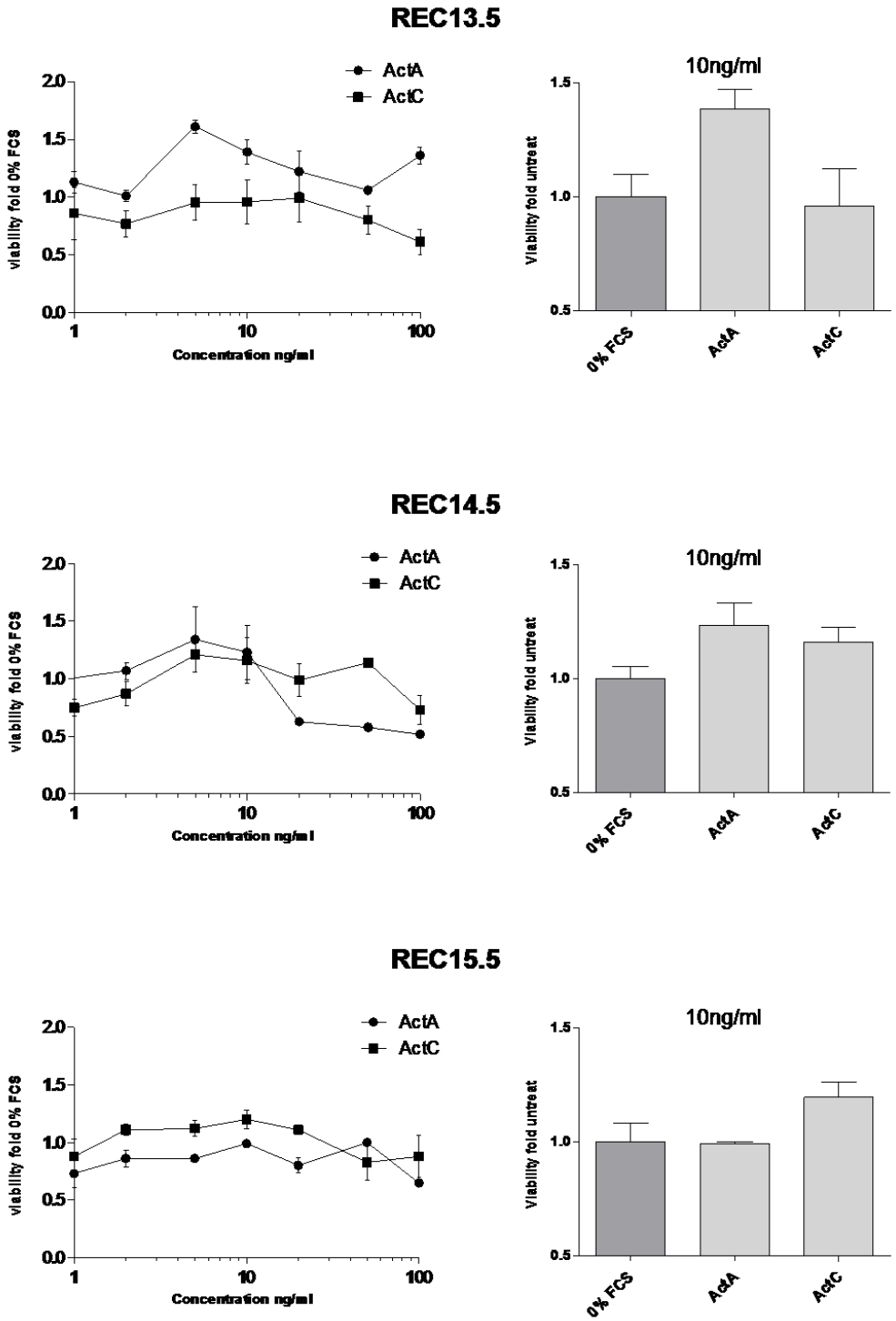

Fig.1 Activin C enhances viability in rat embryonic cells depending on their stage of development

REC cells prepared from 13.5, 14.5 and 15.5 day-old Fischer rat embryos were allowed to attach to 96-well microtiter plates overnight. Cells were exposed to 10ng/ml recombinant human activin A and C under serumfree conditions for 48 hours. Incubation with 30µl of 5mg/ml MTT stock solution at 37°C took approximately 30min. DMSO was used to lyse the cells. Means ± SD are given from 3 separate experiments. Data are expressed as fold 0% FCS.

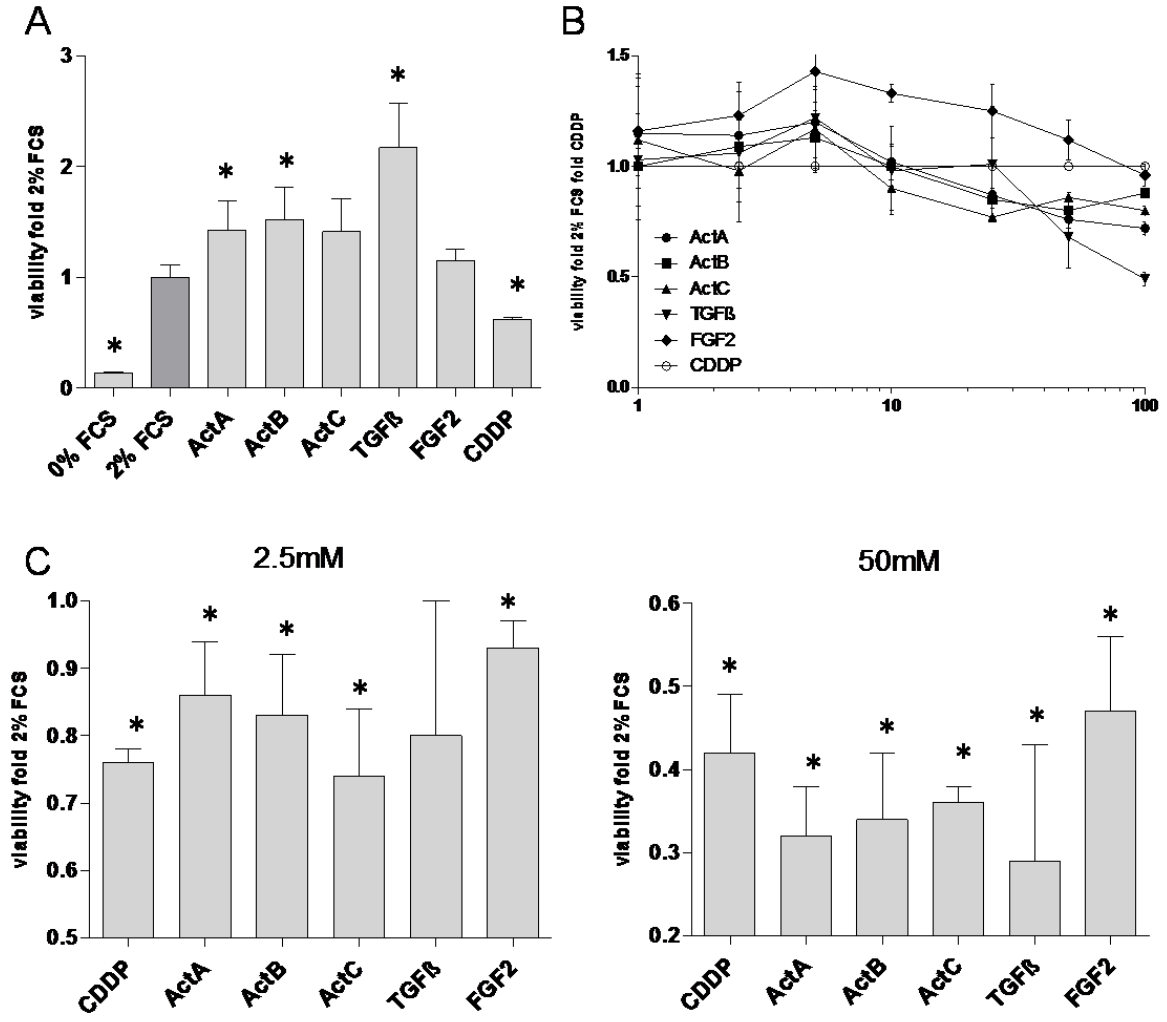

Fig.2 Activin subfamily members modulate viability of 423-cells

To determine the effect of activins on viability of 423-cells a MTT assay was performed. Incubation with 30µl of 5mg/ml MTT stock solution at 37°C took approximately 30min. DMSO was used to lyse the cells. Results of at least three different experiments are given and expressed as means ± SD are. Significant differences were assessed relativ 2% FCS (* $p<0.05$). (A) Cells attached to 96-well microtiter plates were incubated with 10ng/ml recombinant human activin A, B and C and TGFβ under serumfree conditions for 48 hours. 2.5ng/ml FGF2 served as viability enhancing positive control, whereas 10mM cisplatin served as negative control. Data are expressed as fold 0%FCS. (B) Constant 10ng/ml of activins and TGFβ respectively 2.5ng/ml FGF2 were administered to increasing concentrations of cisplatin (CDDP). Concentrations ranged from 1mM to 100mM. Data are expressed as fold 2%FCS fold CDDP. (C) Exemplary two different concentrations of cisplatin treatment are given: 2.5mM and 50mM. Cells additionally were exposed to 10ng/ml of activins and TGFβ respectively 2.5ng/ml FGF2. Data are expressed as fold 2% FCS.

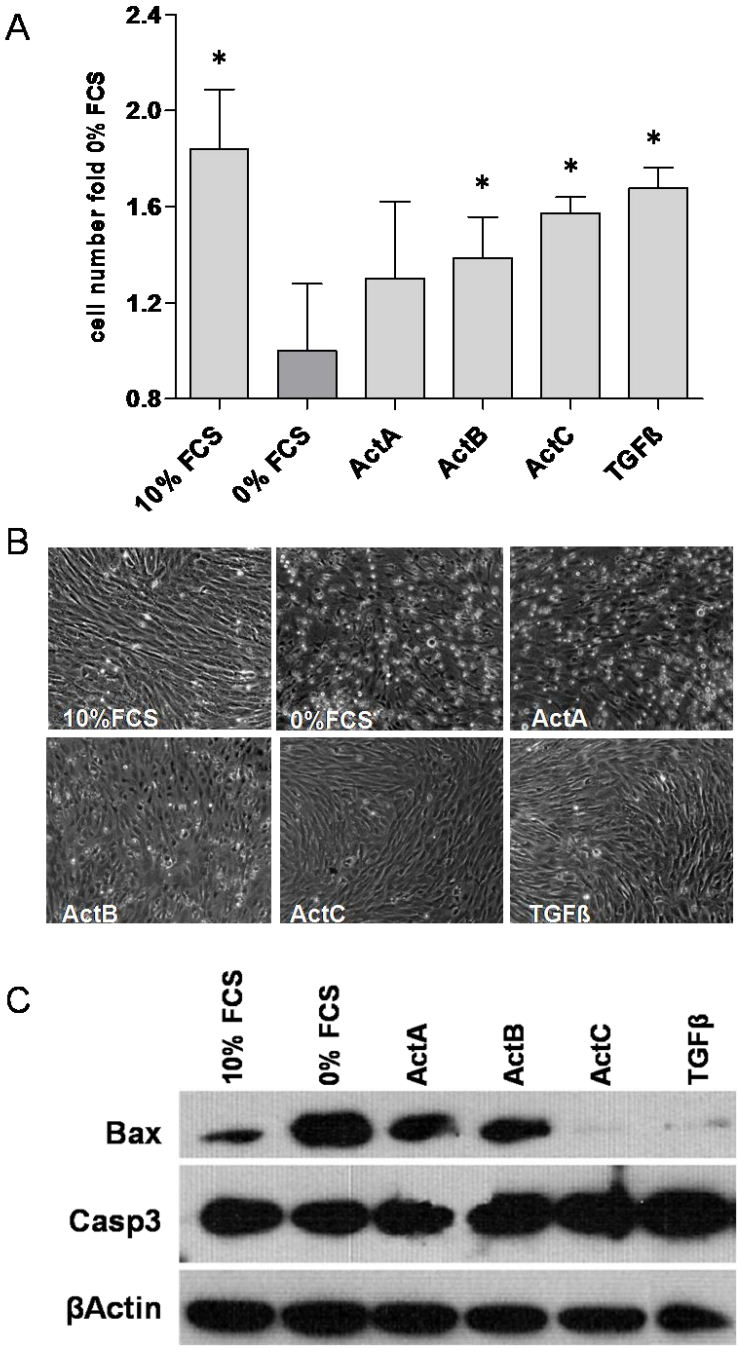

Fig.3 Activin C is a survival factor in 423-cells

423-cells were induced to undergo apoptosis by serum withdrawal and treated with 10ng/ml recombinant human activin A, B and C and TGFβ for 6 hours. (A) Formaldehyde fixed cells were nucleus stained using a 1:1000 dilution of a 5mg/ml DAPI stock solution. Fields in each of the wells were randomly chosen before visual inspection, and at least 300cells/well were counted on a Nikon Eclipse TE 300 microscope. Apoptotic cells were expressed as percentage of the total cell number fold 0% FCS. (B) Differences of rounding up of cells characteristic for apoptosis depending on treatment. (C) Proteins from treated 423-cells were separated by SDS-page and immunoblotted for detection of Bax, Caspase 3 and ßActin. Experiments were repeated at least two times and a representative chemiluminescene blot is depicted. Significant differences were assessed relativ 0% FCS (* $p<0.05$).

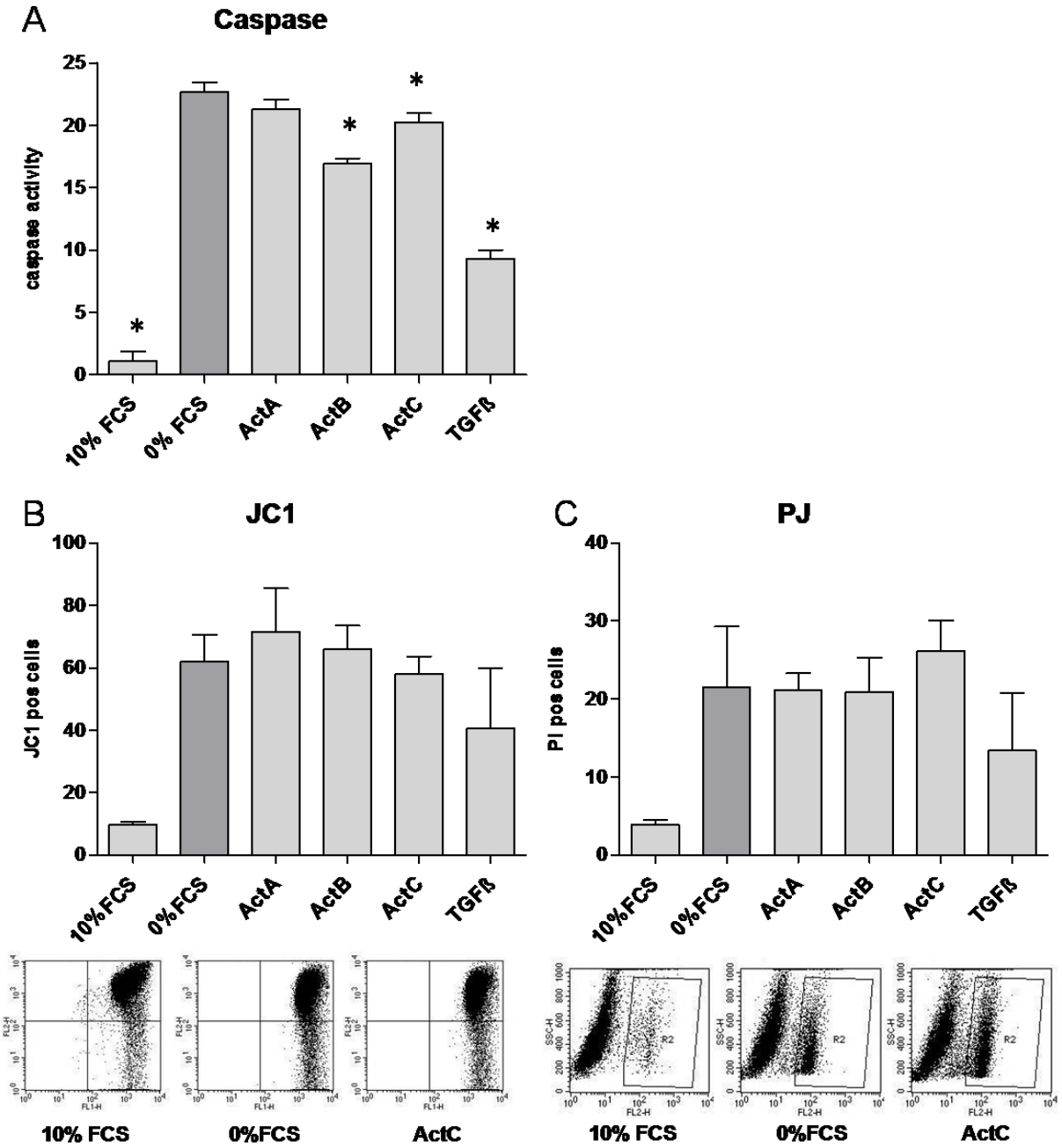

Fig.4 Comparison of different apoptosis assays

Different flow cytometric methods were used to address different stages of apoptosis. 423-cells were seeded and grown to confluence in 6-well plates. Three wells each were treated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in serumfree medium and harvested 24 hours later. (A) The Caspase assay was performed according to manufacturer's introduction. (B and C) For JC1 and PJ detection method cells were harvested through trypsinization, floating and attached cells were combined. 5µM JC1 respectively 10µl of a 30µg/ml propidium iodide stock solution were used as fluorescence dye. Data are expressed as means ± SD representing at least three independent experiments Significant differences were assessed relativ 0% FCS (* $p<0.05$). Typical histograms are given for 10% FCS, 0 %FCS and activin C treatment.

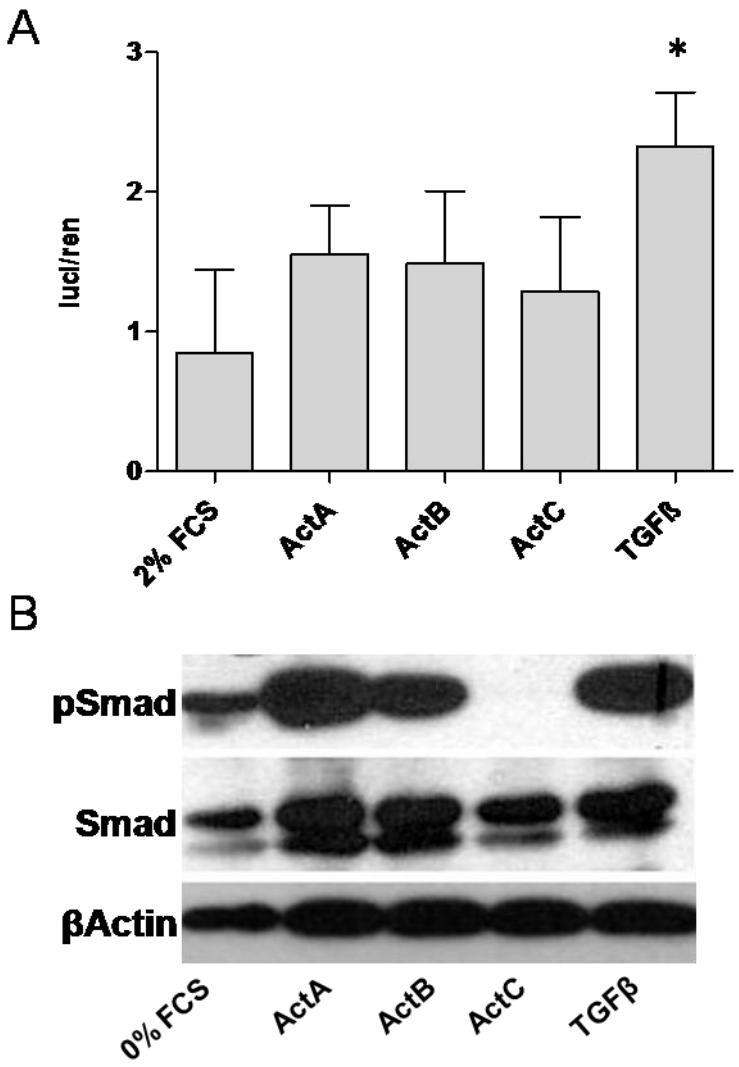

Fig.5 Activin C does not primarily signal via Smad2/3 in 423-cells

(A) 423-cells were transfected with 1µg of the CAGA luciferase construct, consisting of 12 Smad3/4 binding sequences and the luciferase reporter gene. On the next day cells were washed with PBS and treated with 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in serum free RPMI 1640 for 6 hours. Values are expressed as firefly luciferase activity normalized to renilla luciferase activity. Data are means ± SD of at least three separate experiments Significant differences were assessed relativ 2% FCS (* $p<0.05$). (B) For western blot analyses 423-cells were exposed to 10ng/ml recombinant human activin A, activin B, activin C or TGFβ in serum free RPMI 1640 for 30 minutes. Proteins were separated by SDS page and immunoblotted for detection of the phosphorylated and unphosphorylated form of Smad2/3 and ßActin. Experiments were performed at least three times and a representative chemiluminescence blot is depicted.

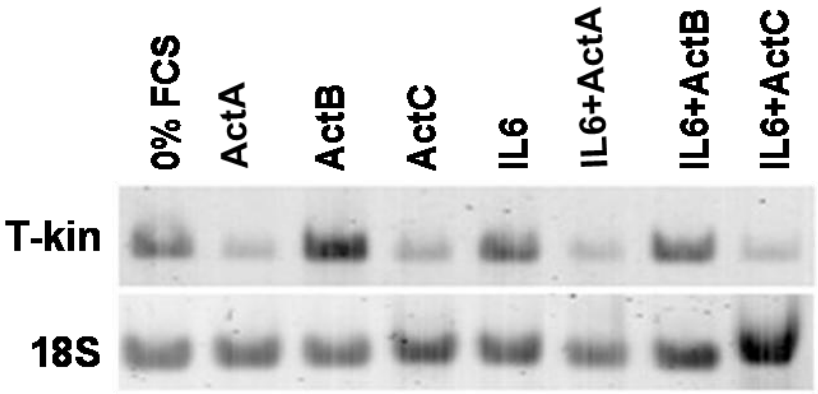

Fig.6 Activin C reduces T-kininogen mRNA levels in primary rat hepatocytes

PRH were cultured in 6-well plates and treated with 10ng/ml of the respective activin, IL6 or a combination of both for 24 hours. RNA isolation, transcription into cDNA and PCR were performed like discribed in material and methods. Also see this section for sequence of primers.

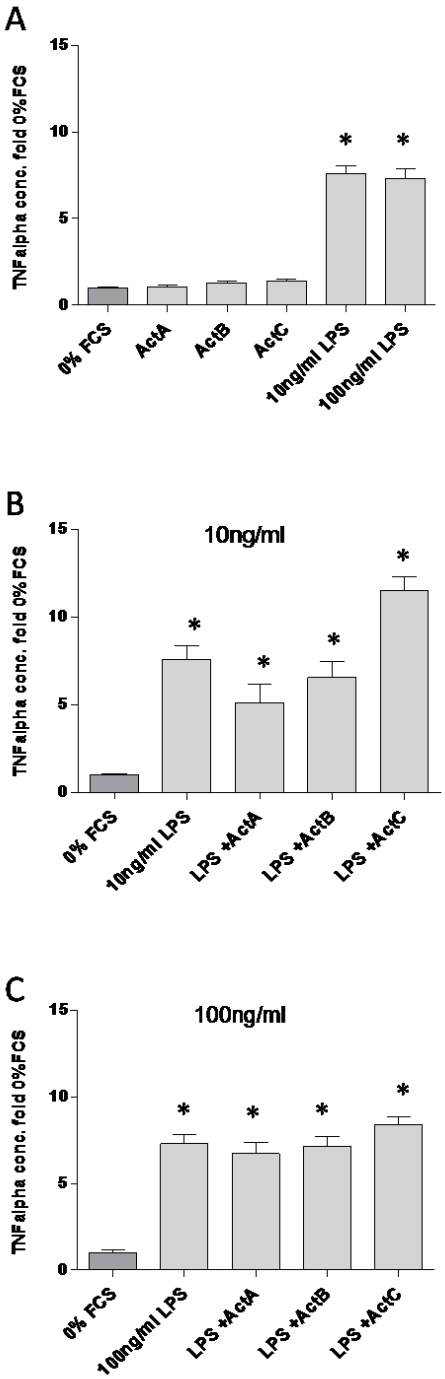

Fig.7 Activin C enhances LPS induced TNFα release of rat kupffer cells

(A) Primary rat Kupffer cells were treated with 10ng/ml recombinant human activin A, activin B or activin C for 48 hours. TNFα levels in PRK supernatants were measured by the rat TNFα Module Set according to the manufacturer´s instructions. (B) PRK were treated with the respective activins combined with 10ng/ml LPS for 48 hours. Supernatants again were analyzed by the TNFα Module Set. (C) LPS concentration was elevated to 100ng/ml. All data are given as means ± SD calculated from at least three different experiments. They are expressed as fold 0% FCS.Significant differences were assessed relativ 0% FCS (* $p<0.05$).

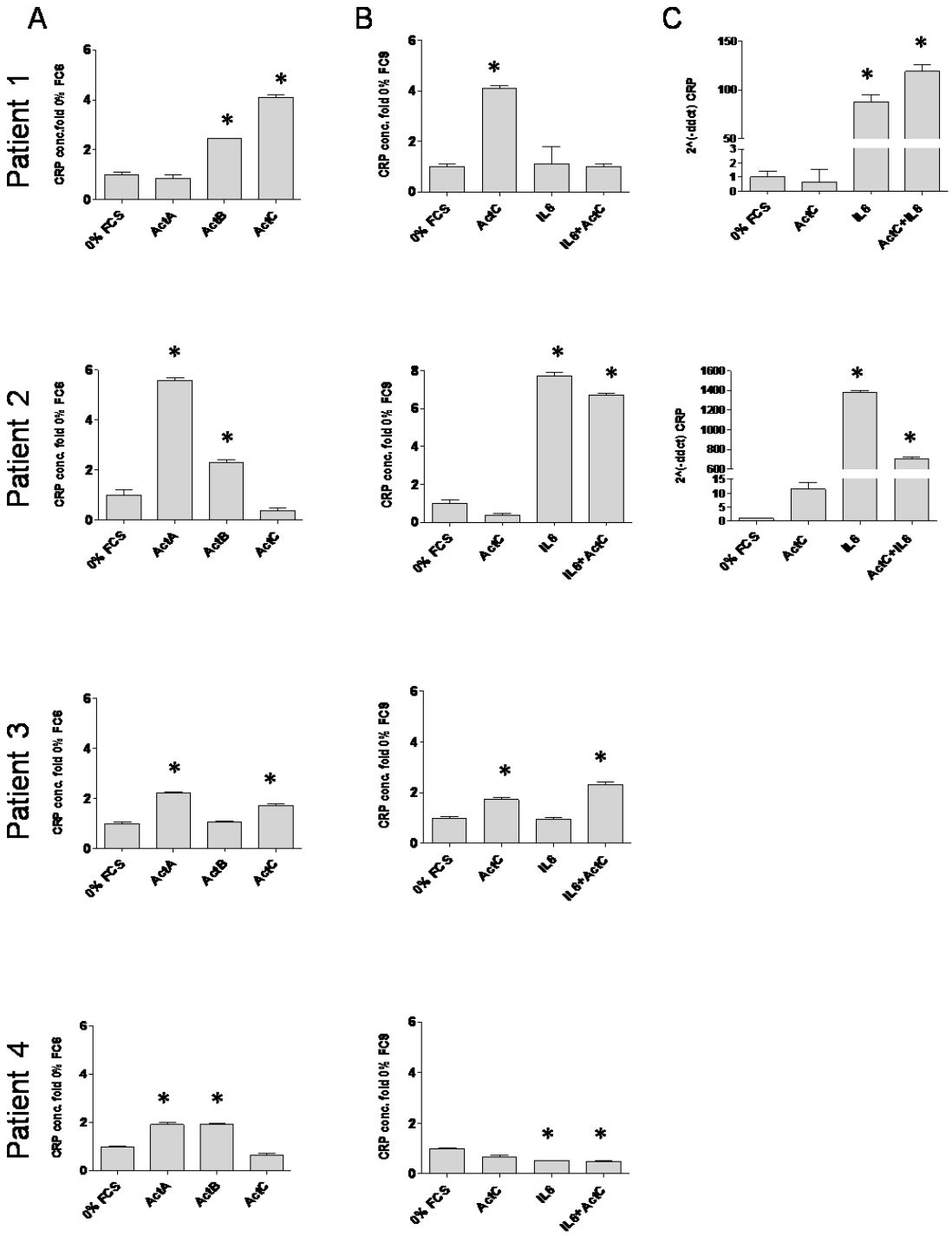

Fig.8 Activins are not suitable as anti-inflammatory agents in humans

(A and B) Human primary hepatocytes were treated with 10ng/ml of the respective recombinant human activins, IL6 ot a combination of both for 48 hours. CRP levels were measured in PHH supernatans by human C-reactive protein Instant Elisa according to the manufacturer´s instructions. Data are means ± SD using triplicates, expressed as CRP concentration µg/µl fold 0%FCS. (C) PRH were cultured in 6-well plates and treated with 10ng/ml activin C, IL6 or a combination of both for 24 hours. RNA isolation, transcription into cDNA and quantitaive RT-PCR were performed like discribed in material and methods. Also see this section for sequence of CRP primers. Data are expressed as 2^(-ddct). Significant differences were assessed relativ 0% FCS (* $p<0.05$).

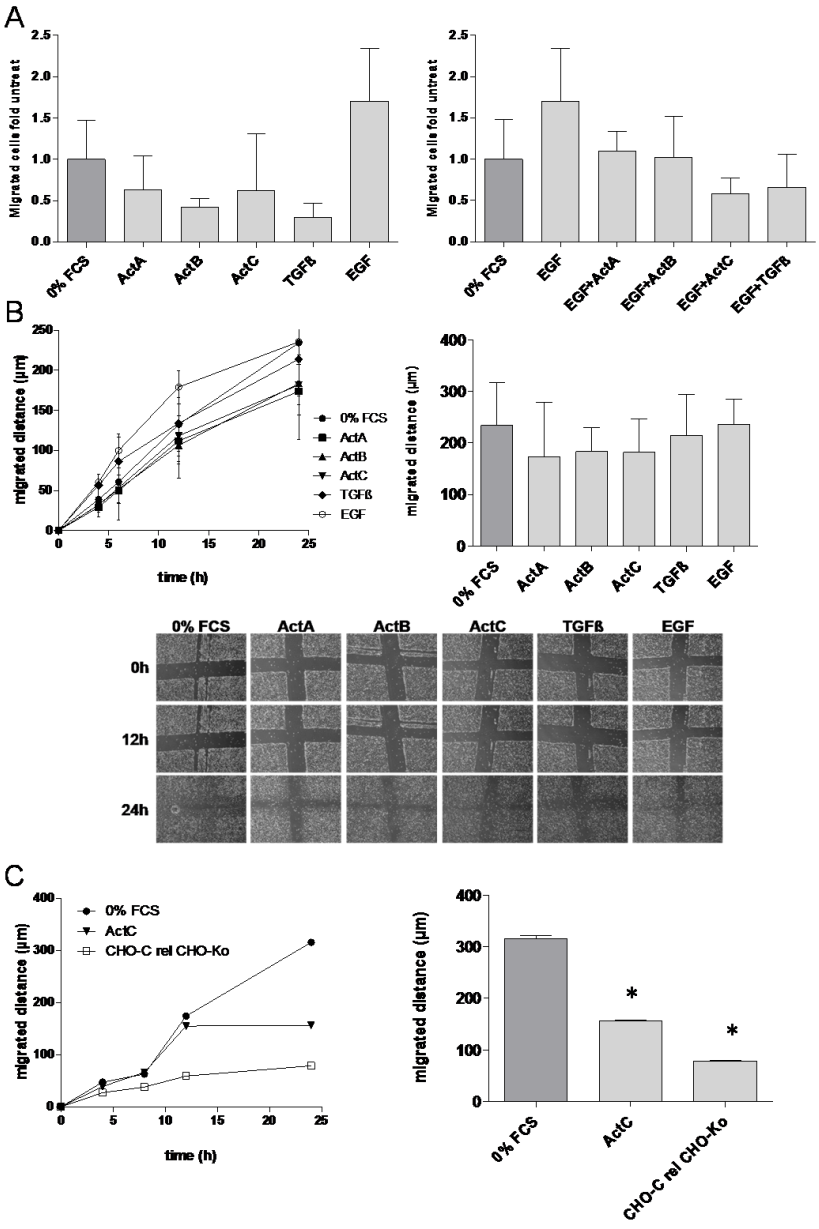

Fig. 9 Reduction of growth factor induced motility by activins in hepatoma cells

(A) A migration assay was performed with $3x10^4$ Hep3b cells under serumfree conditions. They were exposed to 10ng/ml recombinant human activin A, B and C TGFβ for 24 hours. 10ng/ml EGF served as positive control. After 24 hours cells were fixed and nucleus stained as described in material and methods. The whole filter was evaluated and experiments were repeated at least three times. Values are given as mean ± SD fold 0%FCS. (B) Hep3b were grown to a confluent monolayer and scratched. 10ng/ml recombinant human activin A, B and C and TGFβ were added to serumfree RPMI, 10ng/ml EGF served as positive control. Four time points, as representatively depicted, in each scratch were measured during 24 hours and migration distance was calculated from the differences of scratch width. The results are given in micrometer migrated distance (left panel). The right panel represents micrometer migrated at the 24 hours time point and combines the means ± SDs of two respective experiments. (C) Scratched FR3T3 cells were exposed to 10ng/ml recombinant human activin C and to conditioned medium obtained from human activin C producing CHO-C cells. Significant differences were assessed relativ 0% FCS (* $p<0.05$).

Discussion

Activins, members of the TGFβ superfamily, have various effects on many physiological mechanisms, including cell proliferation, cell death, differentiation, inflammation and ultimately also cancerogenicity. While activin A is well investigated and its role in many of the mentioned mechanisms is well established, hardly anything is known about the biological functions of activin C. Hence, this work is an attempt to study the biological function of this neglected subfamily member.

Activins take over important functions of organogenesis and formation of basic structures of solid organs during development (Asashima et al., 1999; Ball and Risbridger, 2001). They are widely expressed during rodent embryonic development, though at different time points and in different tissues. InhβA subunits expression arises on day 10.5 in mesenchymal cells of the developing face, whiskers, hair follicles, heart and digestive tract (Albano et al., 1994; Albano et al., 1993; Feijen et al., 1994; Lu et al., 1993; Manova et al., 1992; Smith et al., 1990; Thomsen et al., 1990). The highly liver specific InhβC subunit expression was detectable at day 11.5 and 14.5 depending on detection method, reaching its maximum at birth (Lau et al., 2000). As the embryonic development is affected by proliferation and apoptosis, we performed viability assays with REC cells, prepared from 13.5, 14.5 and 15.5 day-old Fischer rat embryos. As hypothesized, cells were reactive for added activin A and activin C depending on their stage of development. While activin As positive effect on viability diminished from REC13.5 to REC15.5, activin Cs first showed rather inhibiting effects on viability, but started to enhance it in REC14.5 and REC15.5. Hence, embryonic cells not only express activins at a certain day, but also gain accessibility for these factors not until the day endogenous respective activin is produced. Furthermore these data suggest, that activin A and C effect viability during development depending on the day of growth. As InhβC subunit expression is reported at day 11.5 and 14.5, and our Real Time RT-PCR data revealed only little expression differences in the REC cells, bigger effects may be seen in cells gained from an earlier time point. Comparing cells from different developmental stages regarding Inhβ mRNA expression and reaction on activin treatment together with comparative receptor analysis might be an approach for finding the so far unknown activin C receptor.

Like TGFβ, activin A has been shown to contribute to growth inhibition and apoptosis (Hully et al., 1994; Schwall et al., 1993; Yasuda et al., 1993). Activin B on the one hand inhibits DNA synthesis in primary rat hepatocytes, on the other hand the overexpression of its type I receptor in hepatoma cell lines induces apoptosis (Niimi et al., 2002). Data on activin Cs

effect on cell growth and apoptosis have been rather conflicting (Mellor et al., 2000; Vejda et al., 2003; Wada et al., 2004b). We tried to enlight the effect of the TGFβ subfamily on apoptosis using the embryonic rat cell line 423, a well established apoptosis cell model (Schamberger et al., 2004, 2005). Utilizing six different approaches for measuring survival respectively apoptosis, TGFß was the only used factor always revealing the same effect. Hence, it serves as internal check for the methods. However, the activins turned out to be less stable especially when using flow cytometric analysis.

Nevertheless, we demonstrate that activin C is a survival factor in 423 rat fibroblasts. Upon reduced serum conditions all activins where able to increase viability about 50% compared to control. Whereas in epithelial cells activin A is primarily known to act proapoptotic as seen in hepatocytes and hepatoma cell lines but also in breast cancer cells (Hully et al., 1994; Schwall et al., 1993; Yasuda et al., 1993), in fibroblast there are also reports that credit activin A with mitogenic functions. Hedger et al. for example found FR3T3 to proliferate upon activin A treatment (Hedger et al., 1989; Kojima et al., 1993). This supports our findings in the 423-cells, which are fibroblasts as well. Treatment with increasing concentrations of cisplatin in combination with constant concentrations of 10ng/ml activins and TGFβ, revealed a modulatory nature of activins. Dependent on strength of the previous trigger, they were either able to protect cells from cisplatin induced death, or they contributed to loss of viability. Oberhammer et al. postulated, that priming of cells by other factors is necessary for the apoptotic action of TGFβ, and a similar account may be appropriate for the activins too (Oberhammer et al., 1993).

When monitoring serum starved 423-cells by light microscopy, we found the cells to round up drastically, which are according to Schamberger et al (Schamberger et al., 2004). Addition of activin A did not change this appearance. Activin B treatment however slightly reduced the morphological feature of apoptosis, whereas treatment with activin C and TGFβ had the most positive effect on the cells. All of the added factors enhanced survival when counting the nuclei containing condensated DNA, which reflects viability testing as well as the morphological features. Taken together with a clear reduction of the proapoptotic Bax, activin C and TGFβ turned out to be the most potent survival factors of the TGFβ family members analyzed. In primary hepatocytes or hepatoma cell lines, activin A and activin C are reported to act both proapoptotic (Chen et al., 2000; Schwall et al., 1993). Activin C however is referred to as antiapoptotic as well (Vejda et al., 2003; Wada et al., 2004b). This double edged effect of activin C might be due to a modulatory function of activin C depending on many different factors like experimental setting and previous triggers. On the other hand the differential outcomes might be explained by the fact, that liver cells are the main source of activin C expression, and an autocrine mechanism probably is not the main way of executing its duties. In fibroblast the effect of all the three activins added is relatively the same in three

different detection methods, differing only in the strength of the survival effect induced. Hence, fibroblasts probably are rather the destination of activin Cs properties than endothelial cells.

According to the so far seen survival effects, we found slight reduction of active Caspase 3 upon activin treatment. However, activin C does not cause big differences making a similar Caspase 3 independent mechanism possible as in hepatoma cell lines (see manuscript II). Schamberger et al. showed Caspase 3 to be active in cells while mitochondrial integrity still was maintained and suggest the "point-of-no-return" in death-induced cells to be downstream of activated Caspase 3 (Schamberger et al., 2004). However, flow cytometric detection methods using JC1 to examine changes in mitochondrial membrane potential as an early event in apoptosis and propidium iodide measuring rather late events of apoptosis revealed apoptosis mechanisms upon activin treatment to be more difficult. The former method acknowledges the induction of apoptosis through activin A and B in liver related cells (Hully et al., 1994; Kim et al., 2004; 2002 Schwall et al., 1993; Yasuda et al., 1993), whereas activin C does not show any effect on apoptosis. The second method revealed an apoptotic effect of activin C.

We reason that conditions for detecting activin induced apoptosis are rather difficult, especially when using these TGFß family members without previous triggers. Even in a stable system like the apoptosis model cells 423 determining activins apoptotic functions is unsafe, whereas in this case counting apoptotic nuclei is a suitable choice. All in all, we suggest to always relying on several detection methods.

As investigations on the signaling mechanisms of activin C revealed different signaling modes in the hepatoma cell lines HepG2 and Hep3b (see manuscript II in preparation), we were interested how signaling works in fibroblasts. Determination was performed by Westerblotting, whereas lysates were gained from cells kept under serumfree conditions, and by transcriptional activation assay, whereas cells were kept in medium containing 2% FCS. The different serum levels may explain the different outcomes of the two methods: whereas activin A and B clearly induce phosphorylation of Smad in Westernblot analysis and activin C surprisingly reduces the basal phosphorylation level, the activins hardly show any effect in the luciferase assay. As we already know this phenomenon of measurable and definite effects only under serumfree conditions from other detection methods - for example MTT assays - we suggest that activins are sensitive to serum conditions and their effect is easily diminished by serum contained in medium. Maybe serum deprivation is one trigger to activate the mechanisms of activins. In contrast to the hepatoma cell lines, where activins C did not alter Smad phosphorylation, it reduced the basal state of phosphorylation in 423-cells. However, unlike in HepG2 there was no induction of Smad independent signaling

mechanisms at all. This different outcome of signaling mechanisms in the three differing cell lines emphasizes the setting dependent mode of action of activin C making it even more important to solve this initial step of executing its functions.

Activin A is released rapidly into the circulation during inflammation and modulates several aspects of the inflammatory response. Regulation of the production of cytokines and other immune mediators by activin A is complex, as effects are dependent upon cell type studied, its activation status, the presence of other cytokines, and even the concentration of the activin used (Phillips et al., 2009). We report that activin A as well as activin C reduce basal T-kininogen mRNA levels in primary rat hepatocytes, whereas activin B enhances T-kininogen mRNA expression. Erlach et al., who treated primary rat hepatocytes with inducers of APP followed by human recombinant activin C, revealed alterations in the synthesis of numerous proteins via proteome analysis. These alterations included decreases in secreted APP T-kininogen, fibrinogen β- and γ-chain and fetuin A while synthesis of transthyretin, albumin and apolipoprotein A-1 was increased. Furthermore activin A has been shown to inhibit the induction of APP synthesis by IL6 in the hepatoma cell line HepG2 (Werner and Alzheimer, 2006). This so far known ability of activin A and C to inhibit acute phase protein induction in general and T-kininogen induction in particular, supports our findings that these two activins act in an anti-inflammatory way.

In our hands not only hepatocytes are targets of activins regulating their response to inflammation, but also kupffer cells, which are important contributors to inflammation. Interestingly, none of the activins were able to regulate Kupffer cell characteristic TNFα secretion on their own, not until cells were first triggered with LPS. Treatment with activin A or activin B reduced the LPS induced TNFα secretion, hence the inflammatory response, whereas activin C even enhanced the release of TNFα. Again, we want to emphasize the importance of a trigger necessary to induce the properties of activins. However, activin A has been reported before to induce the expression of TNFα in rat bone marrow derived macrophages (Nusing and Barsig, 1999). As it is well known to exert pro- as well as antiinflammatory effects, this two opposed results may reflect its double-edged abilities. Additionally, acquisition of cells from different organs may announce for the differences. Whereas TNFα hardly occurs in healthy livers, it can be detected in patients suffering from hepatitis B or C virus infections as well as from alcoholic and non alcoholic fatty liver disease, and from HCC (Chen et al., 2003; Crespo et al., 2001; McClain et al., 2004; Nelson et al., 1997; Sheron et al., 1991). All this diseases are accompanied by inflammation, in which TNFα release triggers cascades of other cytokines. As activin C is primarily expressed in the liver, it might contribute to inflammation related diseases in this organ by enhancing TNFα secretion. Furthermore activin C might be indirectly involved in promotion of

hepatocarcinogenesis as TNFα has been shown to be part of this process ,as well as playing a role in tumor angiogenesis, invasion and metastasis (Knight et al., 2000; Wang and Lin, 2008; Wang et al., 2009). Accordingly, activin A and activin B prevent hepatocarcinogenesis, as they rather inhibit LPS induced TNFα secretion.

With respect to human acute phase proteins, investigations are rather difficult, as primary human hepatocytes, in contrast to primary hepatocytes gained from inbred rat strains, are derived from individuals with divert genetical and health backgrounds. Additionally, cultures may contain non-parenchymal cells contributing to inflammation by secretion of several factors which activate hepatocytes, thus affecting the outcome. As expected, activin treatment of primary hepatocytes had different impact on CRP release from cells gained from four different patients. Furthermore, only one patient turned out to be responsive to IL6. We hypothesized, that this may be due to IL6 levels cells were already exposed to in the body, making them insensitive for further activation. Combined treatment of IL6 and activin C again resulted in different effects on CRP release in all tested supernatants. mRNA levels, however did not reflect protein levels, suggesting the factors not only to influence transcription and translation but also secretion.

Considering the known double edged effects of activin A on inflammation, together with the differential effects of activin C apparently depending on the anamnesis of patients, activins again seem to work as modulators. As there have been discussions about the usage of activin C as antitumor treatment, this outcomes change the way of thinking: as the basic state of an individual can never be fully assessed the application of activin C is much too risky regarding its contribution to inflammation and therefore cancerogenisity.

Motility phenomena contribute to regeneration processes, but also to invasiveness of tumors as they enable the cells to migrate into adjacent tissues or transmigrate limiting membranes and extracellular matrices. Epidermal growth factor is one peptide factor enhancing motility of hepatocytes (Hoschuetzky et al., 1994; Hugo et al., 2007; Klymkowsky, 2005; Nelson and Nusse, 2004). TGFβ has been reported to be chemotactic to HepG2 cells, but not to primary hepatocytes, where it after all enhances EGF induced motility (Stolz and Michalopoulos, 1997). In keeping with this concept EGF clearly induced motility of Hep3b cells in our hands, TGFβ however, neither enhanced the chemotactic motility of Hep3b, nor did it display an additive response when administered together with EGF. After activin A, B and C treatment motility dropped to approximately 50% compared to control, and all of them inhibited EGF induced motility. Interpreting these results seems rather difficult as InhβA expression has been shown to increase after partial hepatectomy until liver mass was fully restored (Gold et al., 2005). InhβB and InhβC mRNA expression however were upregulated in regenerating livers (De Bleser et al., 1997; Gold et al., 2003; Kobayashi et al., 2002). Hence, activin A had

obverse effects to activin B and C, whereas they all induced reduction of motility in our hands. It has to be noted, that so far gained data concern livers in vivo and not hepatoma cell lines like it is the case in our study. Thus different modes of actions emerge for activin A on the one hand, and activin B and C on the other hand: InhβA mRNA expression has to be downregulated to allow regeneration of injured livers, and treatment of hepatoma cells with activin A inhibits their motility. Considering its proapototic role, activin A may act as anticancerogen. This is very well supported by antitumorigenic effects seen in other carcinoma tissues. Activin A inhibits proliferation of gall bladder, prostate and pituitary gland cancers cells (Danila et al., 2000; McPherson et al., 1999; Yokomuro et al., 2000). It also is an inhibitor of angiogenesis, therefore preventing tumor progression (Panopoulou et al., 2005). However, there are reports that show tumorigenic effects for activin A as well. It mediates, for example, high N-cadherin expression in human esophageal carcinoma cell surface, which is associated with tumor aggressiveness and poor prognosis (Yoshinaga et al., 2004). Furthermore it increases the proliferation of several ovarian cancer cell lines (Steller et al., 2005). βB and βC mRNA expression is upregulated in livers after partial hepatectomy (Takamura et al., 2005). Yet, their addition to Hep3b cells reduced motility. To complicate interpretations even more data on apoptosis and activin C are conflicting (Mellor et al., 2000; Vejda et al., 2003; Wada et al., 2004b). So two different possibilities arise: it might, similar to activin A, serve as anticancerogen as well, or it might promote cell growth but inhibit metastasis. Hence, activins can exert anticancerogenic or cancerogenic effects, strongly depending on the setting.

All in all this study demonstrates the roles of activins in many physiological mechanisms, and shows that activin C modulates basic pathways and biological functions. Importantly, we put on record, that the activin subfamily, and especially activin C, needs a foregoing trigger to conduct its functions. There will have to be preceding investigations to completely elucidate the interwoven fields of action of these complex TGFβ family members.

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