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DIPLOMARBEIT

The Role of Activin beta E
in Receptor Mediated Signaling

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Abstract

Activin beta E is a member of the TGF-beta family of growth and differentiation factors. It is closely related to the other mammalian activin subunits activin beta A, beta B and beta C. Like them, it is synthesized as a biologically inactive monomeric proform which forms homo- and heterodimers and is processed by endoproteases, producing the mature peptide. The mature homodimer (activin E), or heterodimers containing the beta E subunit (e.g. activin AE) are subsequently secreted by the cell. Activin beta E has been found to be predominantly expressed in the liver, but to be downregulated in hepatocellular carcinoma. Overexpression of the protein in liver cells inhibited DNA synthesis and increased apoptosis. Upon liver damage, expression of INHBE, the gene coding for activin beta E, was downregulated. The role of the protein in mammalian physiology remains unknown, as interaction partners of the protein have not been identified so far and knock-out mice did not show an abnormal phenotype in liver regeneration or development.

In order to investigate the biological functions of activin beta E, we established mammalian and insect cell expression systems, producing various epitope tagged and untagged variants of the protein. While exogenous addition of the prospective signaling molecule did not significantly alter the proliferation of the liver cell lines HepG2 and Hep3B, the producer cell lines themselves showed strongly decreased proliferation and increased cell size.

Treatment of the erythroleukemia cell line K562 with activin A, but not activin E, induced differentiation of the cells towards the erythroid lineage. Also cotreatment with activin A and activin E did not significantly alter the effects of activin A on the cells.

Similarly, incubation of HepG2 cells with supernatants of activin beta A but not activin beta E expressing cells caused phosphorylation of smad 2. Cotreatment with low amounts of activin A and supernatants from activin beta E expressing cells did not noticeably affect the smad 2 phosphorylation due to activin A in comparison to mock supernatants.

While effects on MAP kinase signaling and on other pathways activated by activin A remain to be elucidated, our results indicate that activin E does not signal via similar

pathways as activin A, nor does it inhibit the action of its more famous relative at this level.

Zusammenfassung

Activin beta E gehört zur TGF-beta Familie von Wachstums- und Differenzierungsfaktoren. Wie seine nahen Verwandten in Säugetieren, Activin beta A, Activin beta B und Activin beta C, wird es als inaktive, monomerische, Proform synthetisiert. Diese kann in Folge Homo- und Heterodimere bilden. Ein Homodimer aus zwei beta E Untereinheiten wird Activin E, ein Heterodimer etwa aus einer beta E und einer beta A Untereinheit Activin AE genannt. Nach der Prozessierung durch Endoproteasen wird das mature Dimer sezerniert.

In Säugetieren wird Activin beta E hauptsächlich in der Leber exprimiert. In Lebertumoren ist die Expression jedoch reduziert. Selbiges geschieht im Zuge der Leberregeneration nach einer Schädigung. Künstliche Überexpression führte zu einer Hemmung der DNA-Synthese und verminderter Zellproliferation.

Die physiologische Funktion des Proteins ist jedoch großteils unbekannt, da bisher keine Interaktionspartner nachgewiesen werden konnten, und Knock-out Mäuse keine Veränderungen bezüglich Entwicklung oder Leberregeneration zeigten.

Zur Ergründung der Rolle des Proteins in Säugetieren wurden von uns Expressionssysteme für epitopmarkiere Formen des Proteins etabliert. Behandlung der Leberzelllinien HepG2 und Hep3B mit Überständen von Activin beta E exprimierenden Zellen zeigte keine deutliche Beeinflussung des Wachstumsverhaltens der Zellen. Die überexprimierenden Zelllinien selbst jedoch wuchsen langsamer und hatten einen deutlich größeren Zelldurchmesser als Kontrolllinien.

Die Leukäme-Zelllinie K562 reagierte mit Differenzierung auf die Zugabe von Activin A zum Medium, nicht jedoch auf die Zugabe von Activin E. Ebenso wenig hemmte Activin E die Wirkung von Activin A.

Behandlung von HepG2 Zellen mit den Überständen von Activin beta A exprimierenden Zellen, nicht jedoch mit denen von Activin beta E exprimierenden Zellen, führte zur Phosphorylierung von Smad 2. Die gemeinsame Behandlung der HepG2 Zellen mit Activin A und Activin E führte zu keiner deutlichen Verminderung der durch Activin A hervorgerufenen Phosphorylierung im Vergleich zu Kontrollen.

Obwohl die Effekte von Activin E auf andere Signalwege, welche durch Activin A aktiviert werden, noch nicht untersucht werden konnten, deuten unsere Ergebnisse

darauf hin, dass Activin E deutlich anders wirkt als Activin A und dieses auch nicht in seiner Wirkung inhibiert.

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1. Introduction

Activin beta E is a member of the activin branch of the TGF-beta family of growth and differentiation factors (Schmierer & Hill, 2007) – for an overview see Figure 1. It was first described in the mouse by Fang et al. (Fang, Yin et al., 1996). This was followed by identification and cloning of the orthologue in rat (O'Bryan, Sebire et al., 2000) and human (Hashimoto, Tsuchida et al., 2002). The human genome contains genes encoding for four different activin subunits called activin beta A (encoded by INHBA), activin beta B (INHBB), activin beta C (INHBC) and activin beta E (INHBE) (Deli, Kreidl et al., 2008). Activin beta D has so far been described in *Xenopus laevis* only (Oda, Nishimatsu et al., 1995). The name activin is derived from the original description of the founding member of this protein family as an inducer / enhancer of synthesis and secretion of Follicle-stimulating hormone (FSH) (Harrison, Wiater et al., 2004). Later activins were reported to play a role in multiple other processes like development, erythroid differentiation, nerve cell survival, mesoderm induction in *Xenopus*, bone growth and induction of cell cycle arrest and apoptosis (Woodruff, 1998). The function of activin beta E remains to be elucidated. The following sections will focus on the properties and functions of activins in humans.

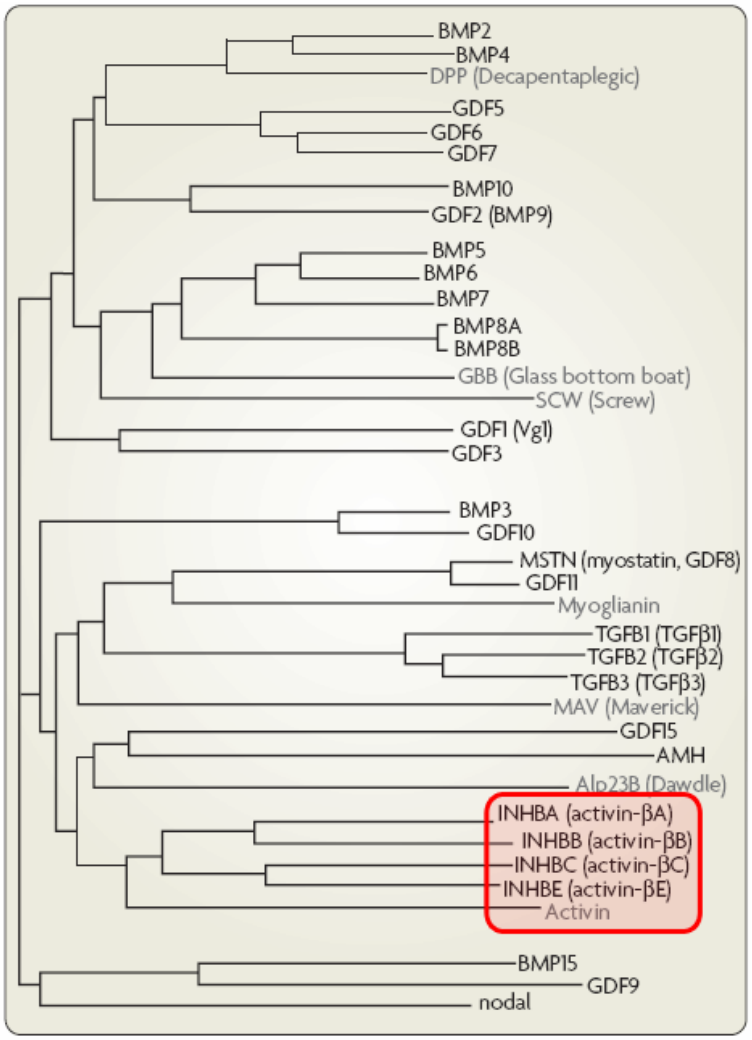


Figure 1 relationships within the TGF-beta family;
phylogenetic tree derived from protein alignments of TGF-beta signaling molecules in humans (black) and *Drosophila melanogaster* (grey); adapted from (Schmierer & Hill, 2007)

1.1. Structure

Activins are homo- or heterodimers of the four different subunits named above. A homodimer of two activin beta A subunits gives rise to activin A, a heterodimer of one beta A and one beta B subunits gives rise to activin AB. While activins AB and AC have been described under endogenous conditions in vivo (Evans, Muttukrishna et al., 1997; Mellor, Ball et al., 2003), activins BC, AE and CE have only been found in in vitro systems so far (Mellor, Cranfield et al., 2000; Vejda, Cranfield et al., 2002; Vejda, Erlach et al., 2003; Wada, Medina et al., 2005). Activin subunits are synthesized as pro-proteins of 350 to 426 aa (Deli, Kreidl et al., 2008). The protein is glycosylated in the prodomain region, but addition of the carbohydrate group seems to be dispensable for secretion. This is in contrast to the related inhibin alpha, a

member of the TGF-beta family and dimerization partner of activin subunits (see below) (Antenos, Stemler et al., 2007).

Dimers are created by intermolecular disulphide bond formation between the sixth of nine conserved cysteines in the mature proteins. The other cysteines are involved in the formation of intramolecular disulphide bonds, creating the so-called cysteine knot, typical for members of the TGF-beta family. It is required for biological activity of the proteins (Mason, 1994).

Following dimerization, the protein is cleaved by pro-protein convertases of the subtilisin/kexin family in ER and Golgi, producing a mature peptide chain of 115 to 116 aa (see Figure 2). While the biologically active protein is secreted as a dimer of the mature peptides only, it has been suggested that the pro-region is required for correct folding, dimer formation and secretion (Gray & Mason, 1990). Unprocessed, dimeric activin A was found to be biologically inactive (Mason, Farnworth et al., 1996). Monomers have been reported to retain some affinity for the receptors of dimeric activin A but do not cause activation (Husken-Hindi, Tsuchida et al., 1994).

In addition to dimerization with another beta subunit, activin beta A and activin beta B can form heterodimers with inhibin alpha, giving rise to inhibin A or inhibin B, both inhibiting FSH release (Ying, 1987). It remains unknown if inhibin C exists, as there was evidence for the formation of a dimer between activin beta C and inhibin alpha in some (Ushiro, Hashimoto et al., 2006) but not in other experiments (Mellor, Cranfield et al., 2000). No data on this subject has been published for activin beta E.

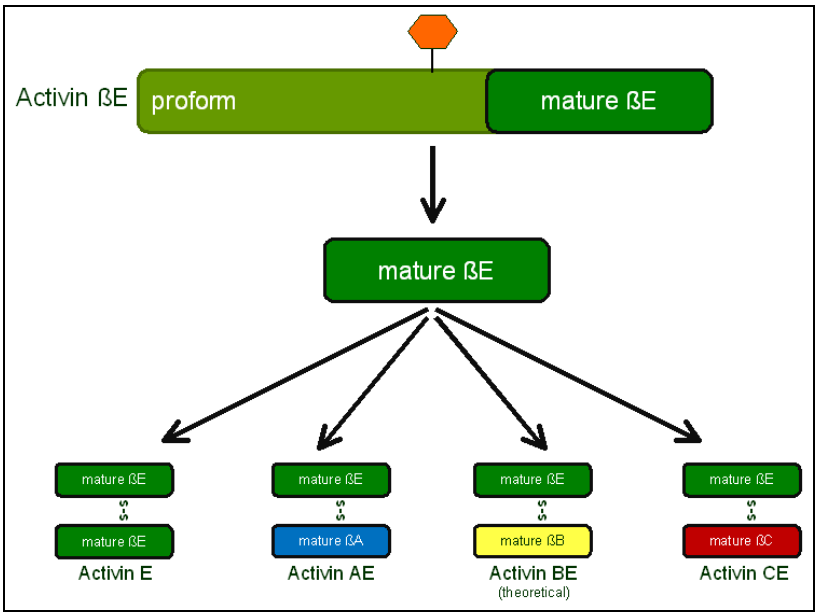


Figure 2 structure and dimerization of activin beta E;
activin beta E is synthesized into the ER as a pro-protein containing a glycosylation signal in its prodomain; it is able to dimerize with other subunits of the activin family via the formation of disulphide bridges and is then processed and secreted

1.2. Expression

While the genes coding for activin beta A and activin beta B, INHBA and INHBB, are almost ubiquitously expressed, mRNA from INHBC is only detected in the liver and (to a lesser degree) in a small number of other organs (Butler, Gold et al., 2005).
INHBE is highly expressed in the liver only (Fang, Wang et al., 1997; Vejda, Cranfield et al., 2002), but has also been detected by RT-PCR in the rat lung (O'Bryan, Sebire et al., 2000) and at low levels in other tissues as well (Hashimoto, Tsuchida et al., 2002; Vejda, Cranfield et al., 2002). The genes coding for activin beta C and beta E are closely linked and are thought to have arisen from tandem duplication of an ancestral gene (Fang, Wang et al., 1997).
Expression of activin beta E mRNA was transiently upregulated following liver damage and downregulated in hepatocellular carcinoma (Grusch, Drucker et al., 2006; Rodgarkia-Dara, Vejda et al., 2006). The latter observation has also been made for activin A which in contrast was found to be downregulated in response to liver damage (Deli, Kreidl et al., 2008). Additionally, INHBE expression was found and confirmed to be elevated in HepG2 cells as a consequence of phospholipidosis, a lipid storage disorder (Atienzar, Gerets et al., 2007; Sawada, Takami et al., 2005).

In the liver of the developing mouse, activin beta E expression could not be detected until the very late stages of embryonic development and peaked at birth (Lau, Kumar et al., 2000).
Large scale analysis identified mutations in the INHBE gene to be significantly increased in breast cancer (Sjoblom, Jones et al., 2006). An evaluation of single nucleotide polymorphisms (SNPs) in genes coding for activins in testicular cancer showed a correlation for the risk of disease and mutations in INHBA but not in INHBB, INHBC or INHBE (Purdue, Graubard et al., 2008).
Overexpression of the tumor suppressor RASSF1A stimulated expression of INHBE, while knock-down of endogenous RASSF1A in nasopharyngeal epithelial cells resulted in downregulation of INHBE (Chow, Lam et al., 2006).
INHBE was also reported to be a target of hedgehog signaling (Katoh & Katoh, 2008).
Finally, in gene chip analysis, mRNA levels from INHBE were found to be altered in HepG2 in response to hypoxia (Fisher, Etages et al., 2005).

1.3. Receptors

Like other members of the TGF-beta family, activins are believed to signal via single-pass transmembrane receptors with an intracellular Ser–Thr kinase domain. This has been proven for activins A, B and AB. Activin A first binds to dimers of the type II receptors ActR-II (aka ACVR2) or ActR-IIB (aka ACVR2B), leading to the (preferential) recruitment and phosphorylation of dimers of the type I receptor ALK4 (aka ActR-IB / ACVR1B). While binding to the same type II receptors, activins B and AB preferentially recruit ALK7 (ACVR1C) as type I receptor (Deli, Kreidl et al., 2008; Schmierer & Hill, 2007). Upon ligand binding, receptors are typically internalized (Dore, Yao et al., 2001). It has been questioned however, if this internalization is always necessary for signaling (Zhou, Scolavino et al., 2004).
As a consequence of activation, receptor-regulated smads (R-smads) are recruited to the receptor complex and phosphorylated by the type I receptor. This process is supported by accessory proteins like SARA and the motor protein kinesin-1. Depending on the identity of this receptor, either smad 2 and smad 3 (ALK4, ALK5, ALK7) or smad 1, smad 5 and smad 8 (ALK1, ALK2, ALK3, ALK6) are recruited and

activated (Schmierer & Hill, 2007). For TGF-beta, it has been shown, that the ligand can recruit different type I receptors, activating different subsets of smads depending on the cell type (Lee, Ray et al., 2008). So far, activins have only been shown to signal through smad 2 and smad 3 (Harrison, Wiater et al., 2004). R-smads then form complexes with the common mediator smad 4 and translocate to the nucleus where, together with cofactors, they are directly involved in regulation of gene expression. Despite this narrowing down of the signals induced by several dozen different members of the TGF-beta family, to just a handful of R-smads, signaling is regulated and "personalized" by a large number of inhibitors and modulators (the most famous ones being the inhibitory smads 6 and 7) and the requirement of co-receptors for some, but not for other ligands (Schmierer & Hill, 2007).

In addition, recent evidence suggests smad independent signaling of activin A via MAP kinases ERK 1/2 and p38 (Murase, Okahashi et al., 2001) as well as the phosphatidylinositol 3'-kinase (PI3K) / Akt pathway (Dupont, McNeilly et al., 2003). Rho and JNK were also found to be stimulated by activin A (Zhang, Deng et al., 2005).

Whether or not these mechanisms also apply to activins C, E or heterodimers of their subunits remains unknown.

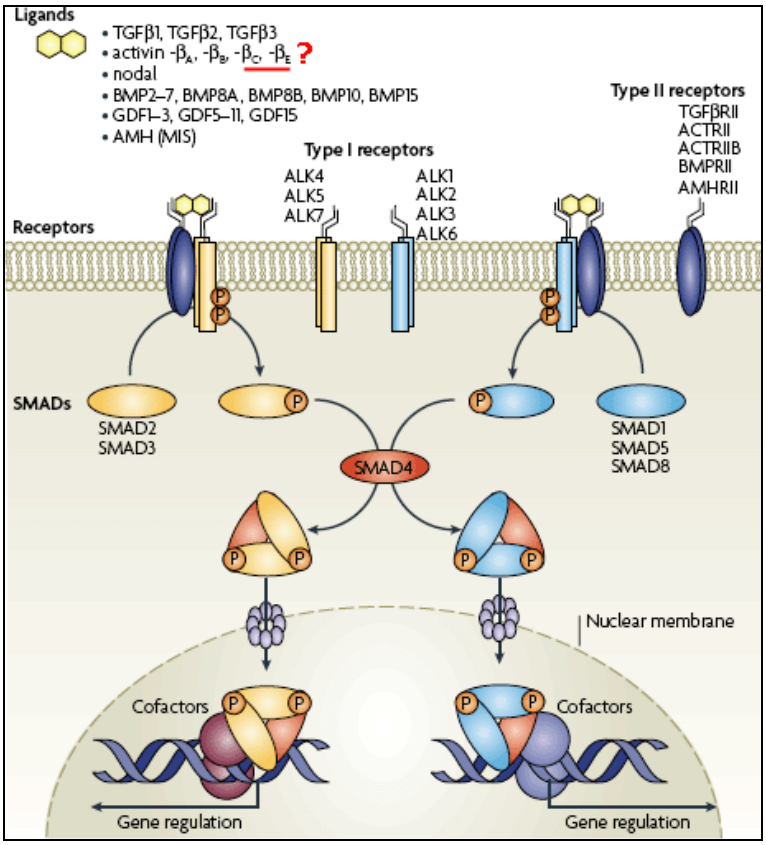


Figure 3 receptor mediated signaling of TGF-beta family proteins via the smad pathway; binding of the ligand to either type I or type II receptors results in the recruitment of the opposite type of receptor and eventually phosphorylation of R-smads; these form complexes with the common mediator smad and translocate to the nucleus where they directly influence gene expression; adapted from (Schmierer & Hill, 2007)

1.4. Biological functions

Both activin A and B have wide-ranging functions in development and tissue homeostasis. Knock-out mice for activin beta A have severe craniofacial defects and die shortly after birth, while deletion of activin beta B results in defects of eyelid development and female reproduction. Cloning of activin beta B into the beta A locus can partially rescue the defects (Chang, Lau et al., 2001). Mice with deletions of activin beta C, beta E or both developed normally and showed no impairment of liver function or regeneration (see below) (Lau, Kumar et al., 2000).

Activin A has been found to stimulate the growth of a variety of cells like fibroblasts, keratinocytes and osteoblasts (Chen, Lui et al., 2002) and is in some cases suspected to aid in carcinogenesis (Chen, Wang et al., 2006). In addition activin A and B were found to act as survival factors for neuronal cells (Kupershmidt, Amit et al., 2007).

On the vast majority of healthy epithelial cells and tumor cells however, activin A acts as a suppressor of proliferation (Risbridger, Schmitt et al., 2001). In hematopoiesis, activin A has been suggested as a negative regulator of lymphopoiesis and an inducer of erythropoiesis (Shav-Tal & Zipori, 2002).
Finally, it has been proposed that effects of exogenous and endogenous activins differ. While some cell lines are immune to the antiproliferative action of activin A in the medium due to production of follistatin, endogenous activin A is able to act as an inhibitor of proliferation in these cells (Delbaere, Sidis et al., 1999).
The biological functions of activin beta E remain largely unknown. Overexpression of activin beta E in the human hepatoma cell lines HepG2 and Hep3B, as well as in the murine hepatocyte cell line AML12 caused decreased proliferation and induced apoptosis (Vejda, Erlach et al., 2003; Wada, Medina et al., 2005). Transient overexpression of activin beta E in the mouse inhibited regenerative DNA synthesis (Chabicovsky, Herkner et al., 2003), while mice constitutively overexpressing the protein showed impaired growth of pancreatic exocrine cells (Hashimoto, Ushiro et al., 2006).
One possible mode of action for activin beta E was described by (Chow, Lam et al., 2006) who showed, that expression of Inhibitor of DNA binding 2 (Id2) is downregulated in response to overexpression of activin beta E. Id2 is a known target of TGF-beta and a potential oncogene (Lasorella, Uo et al., 2001).

1.5. Antagonists

Activin signaling is counteracted by a large variety of intra- and extracellular mechanisms. On the intracellular level, most widely known inhibition happens through the action of the inhibitory smads 6 and 7 (I-smads). In particular for smad 7, the antagonistic effect has been well documented (Lebrun, Takabe et al., 1999). While the exact mechanism of action of I-smads remains unknown (Schmierer & Hill, 2007), it was shown that smad 7 interferes with the association of the receptor smads with the type I receptor ALK4 in case of activin A, by this preventing relaying of the signal to the nucleus (Lebrun, Takabe et al., 1999).

Inhibitory smads may also act by a variety of other mechanisms, like the recruitment of ubiquitin ligases (e.g. SMURF 1 and SMURF 2) or phosphatases (e.g. PP1) to the receptors (Schmierer & Hill, 2007).

Further upstream, activin A signaling is regulated by membrane (bound) proteins. Cripto attaches to the outer membrane via a GPI-anchor and associates with the type I receptor ALK4, acting as a coreceptor for the TGF-beta family protein nodal. Additionally, together with activin A it may associate with type II receptors (ACVR2 or ACVR2B), inhibiting the formation of functional signal complexes (Gray, Harrison et al., 2003). The protein lefty on the other hand has been shown to interact with cripto, preventing signaling by nodal, but allowing signaling by activin A (Cheng, Olale et al., 2004). Cripto has been shown to be overexpressed in several human malignancies. The protein BAMBI which was also found to be highly expressed in some tumors is a pseudoreceptor related to type I receptors, but without a kinase domain, binding to type II receptors and inhibiting signaling. ARIPS 1 and 2 were described to interact with the type II receptor and inhibit activin A signaling (Liu, Tsuchida et al., 2006).

Extracellularly, activins (shown for activins A, B, AB and E (Hashimoto, Tsuchida et al., 2002)) and other TGF-beta family molecules are bound and antagonized by follistatin (Deli, Kreidl et al., 2008). The related protein FLRG acts in a similar manner (Tsuchida, Arai et al., 2000) and has been found to prevent the growth inhibitory actions of activin A in some tumor cells (Razanajaona, Joguet et al., 2007).

Alpha 2-macroglobulin (Vaughan & Vale, 1993) as well as several other proteins have been described to specifically bind activins. It is not in all cases clear however, if this binding serves an antagonistic purpose (Harrison, Wiater et al., 2004).

A completely different mode of regulation happens on the level of dimer formation. As mentioned above, the classic inhibitors of activin A and B, inhibins, have one of their two subunits in common with their counterparts, but employ a different dimerization partner (inhibin alpha). This allows them to bind to the same receptor, without causing a signal. The (competitive) formation of activin AC has been suggested as one mechanism by which activin A signaling is modulated (Mellor, Ball et al., 2003). If activin beta C and E play a similar role, and if beta E also participates in dimer formation with inhibin alpha remains to be elucidated (Phillips, 2000). Figure 4 summarizes several modes of regulation of activin signaling.

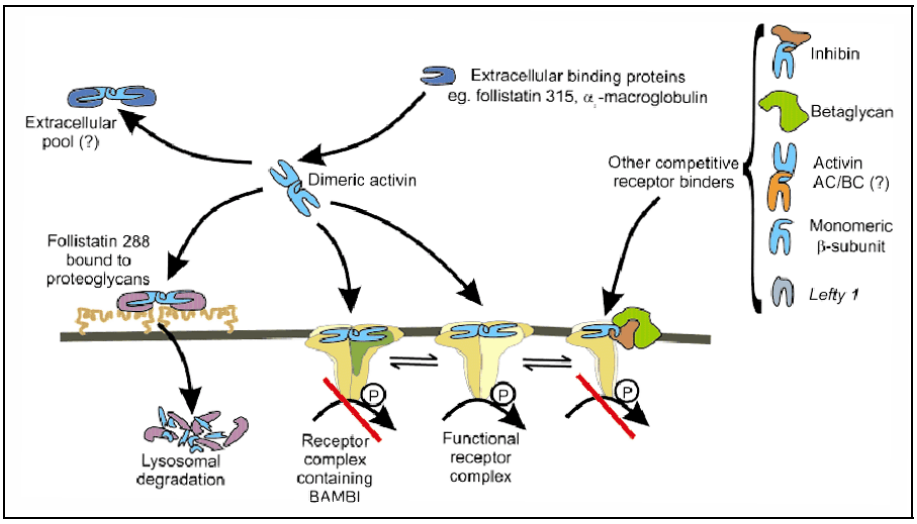


Figure 4 regulation of the action of activins;
activin signaling has been suggested to be regulated – among other means – by extracellular binding of the ligand, competitive receptor binding and reduction of the pool of available monomers for one activin by the formation of other activins containing the same subunit (Phillips, 2000)

1.6. Aims

The aim of this thesis was to elucidate potential functions and signaling mechanisms of the human activin beta E subunit. Central to this was the creation and characterization of expression systems for the study of this little recognized member of the TGF-beta family, as well as the establishment of appropriate in vitro assays for testing its role in signal transduction. While full characterization of the protein would clearly have been beyond the scope of a diploma thesis, setup of functional assays and preliminary results guiding the way for future experiments has been achieved.

2. Materials and Methods

2.1. Cell lines and media

All cell lines (see Table 1) were supplied by the cell culture unit of the Institute of Cancer Research except for CHO DHFR$^-$ cells which were supplied by Biopharm. All media used (see Table 2), except for *MEM alpha* and *Insect Express* were purchased from Sigma. *MEM alpha* was from Invitrogen, *Insect Express* was from PAA. Fetal bovine serum (FBS) and penicillin / streptomycin (P/S) were bought from PAA, hypoxanthine (HT) was from Invitrogen.

Table 1 cell lines used in experiments

cell line		**medium**
CHO-DHFR$^-$	chinese hamster ovary cell line deficient for dihydrofolate reductase (DHFR) (Kingston, Kaufman et al., 2002)	DMEM; 1 x HT* / MEM alpha; 1% P/S
HCT116	human colonic carcinoma cell line (Brattain, Fine et al., 1981)	MEME; 10% FBS; 1% P/S
HEK293	cell line derived from human embryo renal cortical cells (Graham, Smiley et al., 1977)	DMEM; 10% FBS; 1% P/S
Hep3B	human hepatoma cell line (Knowles, Howe et al., 1980)	RPMI; 10% FBS; 1% P/S
HepG2	human hepatoma cell line (Knowles, Howe et al., 1980)	MNP; 10% FBS; 1% P/S
HT1080	human fibrosarcoma cell line (Rasheed, Nelson-Rees et al., 1974)	M+N; 10% FBS; 1% P/S
K562	human myelogenous leukemia cell line (Lozzio & Lozzio, 1979)	RPMI; 10% FBS; 1% P/S
MGC	human glioblastoma cell line (Ponten & Macintyre, 1968)	MEME; 10% FBS; 1% P/S
Sf9	ovarian cell line from *Spodoptera frugiperda* (Vaughn, Goodwin et al., 1977)	Insect Express; 10% FBS; 1% P/S
* before transfection with DHFR plasmid		

Table 2 media used in cell culture

DMEM	Dulbecco's modified Eagle's medium
RPMI	Roswell Park Memorial Institute medium
MEME	Minimum essential medium Eagle
MNP	MEME; 1 mM sodium pyruvate (Sigma); 1% non-essential amino acids (PAA)
M+N	MEME; 1 mM sodium pyruvate
MEM alpha	Minimum essential medium (MEM) alpha modification; medium without (deoxy)ribonucleosides
Insect Express	insect cell medium

2.2. Standard growth conditions

All mammalian cells were grown in a humidified incubator (Forma Scientific) at 37°C; 5% CO_2. Insect cells were grown in closed bottles at 27°C (incubator: Napco). 25 cm^2 (with and without filter), 75 cm^2 and 175 cm^2 tissue culture flasks (T25, T75, T175 respectively) were bought from Greiner Bio-One and BD Falcon. 15cm tissue culture dishes were bought from Sarstedt, 6-well, 12-well and 24-well plates were bought from IWAKI and 96-well plates were from TPP.

2.3. Splitting

For splitting of adherent cells, medium was aspirated and cells were washed once with PBS (137 mM NaCl (Merck); 2.7 mM EDTA (Merck); 10 mM Na_2HPO_4 (Merck) pH 7.4). Trypsin/EDTA (T/E; 0.1% Trypsin (Difco); 0.01% EDTA (Fluka)) was then added and the cells were incubated at 37°C for about 5 min. The cells were then resuspended in medium or in PBS using a pipette. The desired number of cells was then seeded in full medium (medium with serum).

2.4. Freezing

For freezing, cells were grown in T75 bottles in full medium (including selection marker for stably transfected cell lines) until they were almost completely confluent. Adherent cells were detached using T/E and resuspended in 6-8 mL of medium with 10% serum and DMSO (Amresco) at a final concentration of 50 µL/mL. 1 mL each

was transferred to cryotubes (Greiner) and cooled down for at least 24 h in a styrofoam container placed at –80°C before being transferred to a liquid nitrogen tank for long term storage.

2.5. Transfection

Adherent mammalian cells were mainly transfected using either *ExGen 500* (Fermentas) or *FuGENE 6* (Roche) according to the instructions of the manufacturers.

In brief, for a T25 bottle, 6.25 µg of plasmid DNA (one or several plasmids) were diluted in 150 mM NaCl (500 µL total volume), mixed with 20.6 µL of *ExGen* and incubated for 10 min at RT. The mixture was then added to cells in logarithmic growth in full medium. Gene expression was assessed 24-48h following transfection.

In case of *FuGENE 6* (T25), 7.5 µL were diluted in 250 µL of serum free medium and incubated for 10 min at RT. 2.5 µg of plasmid DNA were added and the mixture was incubated for 15-45 min before being added to cells (full medium).

Sf9 cells were transfected using *FuGENE 6* or *Cellfectin* (Invitrogen).

FuGENE was applied in a similar manner as for mammalian cells only that about 5 µg of bacmid DNA were used instead of 2.5 µg.

For transfection with *Cellfectin*, 3 µg of bacmid DNA were diluted in a total of 200 µL serum free medium. 30 µL of *Cellfectin* were mixed with 170 µL of serum free medium which was mixed with the serum / DNA solution. The whole solution was incubated for 45 min at RT and then topped up to 2 mL with medium containing 10% FBS. Old growth medium was removed from the cells to be transfected and the transfection mixture was added to the cells. The following day, a change of medium was performed.

2.6. Selection of stable transfectants

For creation of stably expressing cell lines, the antibiotics neomycin / G418 and puromycin were used to select for cells that had integrated the plasmid coding for the gene of interest and a selection marker into their genome. To reduce the risk of the appearance of fake clones, in most cases a plasmid encoding for a bicistronic mRNA containing the gene of interest and a selection marker, separated by an internal ribosome entry site (IRES; (Gurtu, Yan et al., 1996)), was used. In case of CHO cells, the DHFR system (see 2.7) was used in addition.

The day following transfection, cells were split 1:4 and either G418 or puromycin was added to the cells (see Table 3). About 48 h later, the medium was changed to remove dead cells. At the appearance of resistant clones (1 week following transfection), cells were re-seeded to facilitate growth. Gene expression was assessed by western blot or immunohistochemistry (IHC).

Table 3 additives to media used for the selection of stable mammalian cell lines

cell line	G418 (PAA)	puromycin (PAA)	methotrexate (Sigma)
CHO*	500 µg/mL	5 µg/mL	5 nM (starting concentration)
HEK293	500 µg/mL	0.5 µg/mL	-
*(Asada, Honda et al., 2006; Kingston, Kaufman et al., 2002)			

2.7. Amplification of a gene of interest in stable cell lines

To induce high expression of the gene of interest in stable cell lines, the dihydrofolate reductase expression system (Kingston, Kaufman et al., 2002) was used. In brief, CHO-DHFR$^-$ cells were co-transfected with the respective vector and a plasmid encoding for dihydrofolate reductase (DHFR). This particular cell line, deficient for DHFR, is not able to synthesize certain amino acids and purines if not artificially supplied with them through the medium. Growth in medium not containing nucleosides, like *MEM alpha*, results in selection for cells that have taken up and integrated into their genome, the gene coding for DHFR. Since two plasmids typically integrate into the genome at the same site (Chen & Chasin, 1998; Colbere-Garapin, Ryhiner et al., 1986), it is likely, that the plasmid coding for the gene of interest,

which is supplied in a 5:1 ratio, co-integrates with the DHFR gene. Following transfection, CHO cells were grown in MEM alpha, containing either G418 or puromycin for selection (see above). Once a cell line had been established that grew stably under these conditions and expressed the gene of interest, methotrexate (MTX) (starting concentration 5 nM; see Table 3) was added to the medium. MTX is a competitive inhibitor of DHFR. To adapt to the presence of this inhibitor, cells often react by a gene amplification of the DHFR gene (Banerjee, Ercikan-Abali et al., 1995) which often leads to co-amplification of the gene of interest, integrated nearby. As soon as cells had adapted to these conditions and resumed growth at a normal rate, the concentration of MTX was raised again by a factor of 4. The whole procedure was repeated over the course of several months.

2.8. Immunohistochemistry

For immunohistochemistry (IHC), cells were seeded in 12-well plates containing coverslips (Paul Marienfeld GmbH) 24 h (no further treatment necessary) or 48h-72 h (if cells had to be transfected) prior to staining at a density that resulted in about 75% confluency on the day of staining.

Because of the better results, in all late experiments, cells were fixed with paraformaldehyde (PFA; Fuka) rather than methanol (MeOH; Merck) – see Figure 5. A 37% stock solution of PFA was prepared by dissolving 1.85 g PFA in 3.5 mL water with 10 µL 10 M KOH at 65°C. The stock solution was stored at –20°C. On the day of staining, the medium was aspirated from the wells and the cells were washed twice for 5 min with cold PBS. PBS was removed and the cells were fixed either with PFA (3.7% in PBS) for 10 min at RT or with MeOH for 3 min at –20°C. The fixative was aspirated and cells were washed 3 times for 5 min with PBS. Cells were blocked with PBS, 2% milk for 1 h at RT on a shaker. In case of PFA fixation, 0.5% Triton X-100 (Serva) was added to the blocking solution to permeabilize the cells. Cells were incubated in primary antibody (for list of antibodies used, see Table 4) diluted in blocking solution by putting the coverslip upside-down in the antibody solution and incubating in a wet chamber at 37°C for 1 h. Coverslips were then washed several times in PBS and incubated with secondary antibody in a similar manner. Following

final washes, positive cells were stained by incubating coverslips with DAB solution (DAKO).

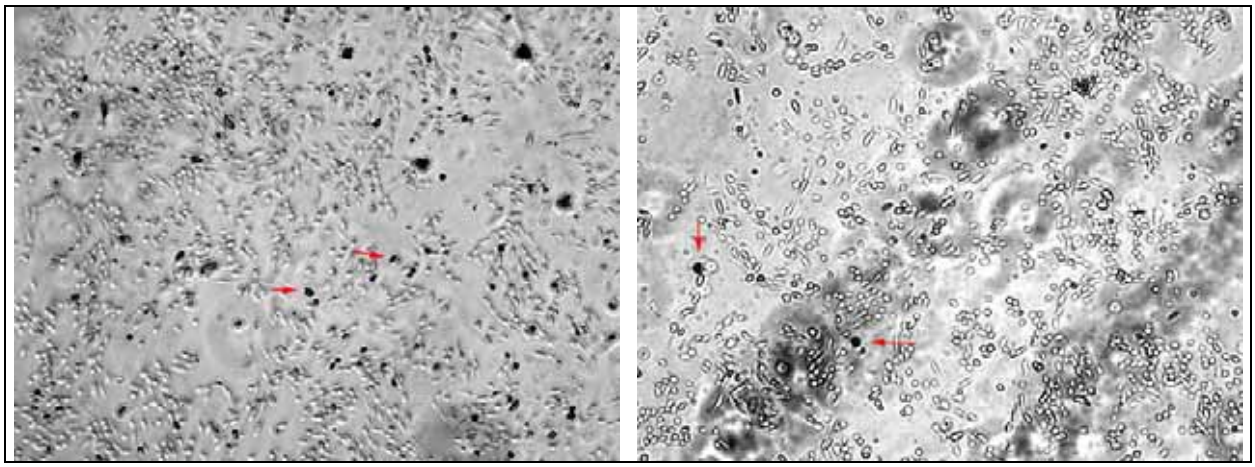


Figure 5 comparison of paraformaldehyde (left) and methanol (right) fixation for IHC; CHO cells, transfected with vector containing an expression cassette for HA-tagged TGF-alpha were fixed either with paraformaldehyde or methanol and stained with rat-anti-HA (1:50) and goat-anti-rat-HRP (Pierce; 1:200); red arrows mark examples of stained cells

Table 4 antibodies used in IHC

mntibody	**dilution used**	**manufacturer**
rat-anti-HA	1:50	Roche; 11867423001
goat-anti-rat-HRP	1:200	Pierce; 31470
rabbit-anti-rat-HRP	1:50	Dako; P0450

2.9. Cell counting

Cells were counted using either a Neubauer chamber (Phelan, 2007) (Paul Marienfeld GmbH) or a Casy cell counter (Schärfe).

2.10. Benzidine staining of K562 cells

K562 cells were seeded in 96-well plates at a density of 10 000 cells per well in RPMI.

As a positive control for the induction of differentiation towards the erythroid lineage, hemin (Ueki, Zhang et al., 2004) (Sigma; final concentration in medium: 50 µM) and

activin A (Wang, Huang et al., 2008) (R&D; final concentration: 25 ng/mL) were added. To determine the effect of activin E, supernatants from cells producing different variants of the protein were added to the cells, alone or in combination with activin A. Experiments were done in triplicates. It was found that the presence of serum in the medium significantly improved the viability of the cells making counting of positive cells significantly easier. Cells were incubated without media change for 3-5 days before staining cells for hemoglobin with 3,3'-dimethoxybenzidine (Lebrun & Vale, 1997). Medium was aspirated and cells were resuspended in 100 µL PBS. 40 µL were transferred to a new well, followed by centrifugation at 1000 g for 5 min. 10 µL benzidine staining solution (1.5 mg 3,3'-dimethoxybenzidine (Dr. Ehrenstorfer GmbH); 1 mL H_2O; 25 µL 30% H_2O_2 (Sigma) 50 µL glacial acetic acid (Merck)) were added directly before evaluation by light microscopy. Digital images were taken and the total cell number was determined automatically using *Image J* software and the *Nucleus Counter* plug-in (http://rsb.info.nih.gov/ij/). Positive cells (see Figure 6) were counted by hand using *Image J* and the *Cell Counter* plug-in.

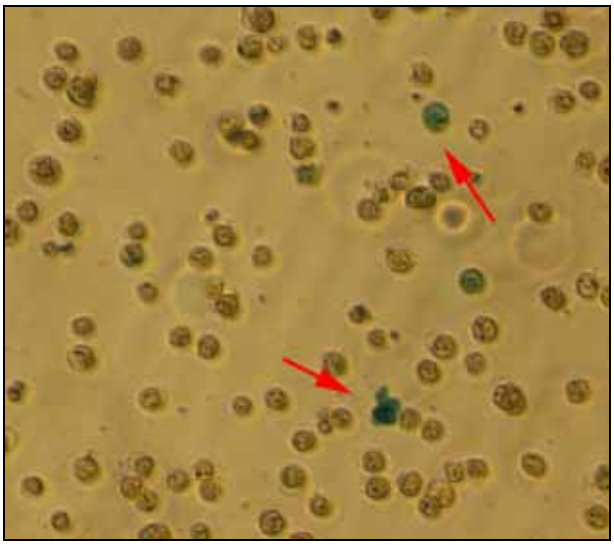


Figure 6 K562 cells stained with benzidine for hemoglobin;
red arrows mark examples of positive cells

2.11. Determining DNA concentration

Concentration of purified linear or circular DNA was determined using a *NanoDrop 1000* spectrophotometer (Thermo Fisher Scientific).

2.12. Restriction digest

Restriction digest was performed (where possible) in 10 µL total volume. For larger volumes, amounts were increased accordingly. To determine the identity of a plasmid, about 200 ng of DNA were digested with 5 units restriction enzyme (Fermentas, unless stated otherwise) in respective buffer for 2-3 h at the suggested incubation temperature (typically 37°C). For preparative digestion 1-3 µg of plasmid were digested with 10-20 units of restriction enzyme overnight (o/n).
For double digests, buffers and conditions (excess of one enzyme over the other) were used as suggested by Fermentas.
At several occasions, Fermentas *FastDigest* buffer was used (for *FastDigest* and regular enzymes) and incubation time was reduced to 15-30 min. In general, conventional digestion seemed to yield better results (data not shown).

2.13. Dephosphorylation of linear DNA fragments

To prevent recircularization of a vector backbone without the integration of an insert (where desired), linear DNA was dephosphorylated with calf intestinal alkaline phosphatase (CIAP; Fermentas). 1 µg of DNA was incubated with 1 unit of CIAP either in the buffer supplied with the phosphatase or any restriction enzyme buffer for 30 min at 37°C. Incubation temperature was raised to 50°C and CIAP was added again at 1 unit per µg of DNA. Incubation was continued for another 30 min before the reaction was terminated by increasing the temperature to 85°C for 15 min or purifying the DNA by agarose gel electrophoresis.

2.14. Phosphorylation of linear DNA fragments

For direct integration of PCR products into a blunt end vector backbone, the PCR product generated by Pfu DNA Polymerase was phosphorylated using T4 polynucleotide kinase (PNK; Fermentas). 50 µL PCR reaction were mixed with 5 µL PNK (10 units/µL), 16 µL PEG 6 000 (Fermentas), 9 µL 10 x *PNK-Buffer A* and 9 µL rATP (10 mM; Promega). The reaction was incubated at 37°C for 1 h and

stopped either by increasing the temperature to 75°C for 10 min or purification of the DNA fragment by gel electrophoresis.

2.15. Gel electrophoresis

DNA fragments of the typical size of 1-3 kilobases (kb) were separated on gels consisting of 1% (w/v) agarose (Biozyme) in 0.5 x TBE buffer (5.4 g/L Tris (Fluka) 2.75 g/L boric acid (Sigma); 1 mM EDTA). Samples were mixed with 6 x loading buffer (333 µL/mL 6 x loading dye (Fermentas); 250 µL/mL 80% glycerol (Merck); 66.5 µL/mL 0.5 M EDTA; 0.5 µL/mL 10 000 x *Vistra-Green* (GE Healthcare)) and loaded onto the gel. As a marker 2 µL *GeneRuler 1 kb DNA Ladder* (Fermentas), mixed with appropriate amounts of water and loading buffer were used. 0.5 x TBE was used as a running buffer and the gel was run at a constant voltage of 50 V for 10 min, followed by 160 V for 30 min (power supply: Bio-Rad).
For smaller fragments, 2% gels (marker: *GeneRuler 100 bp Plus DNA ladder* (Fermentas)), and for larger fragments 0.5% gels were used.
Bands were visualized using a *FluorImager 595 Scanner* from Molecular Dynamics.

2.16. Ethanol precipitation

Precipitation of DNA (e.g. for sequential digest using different buffers) was performed by mixing DNA solution with 3 volumes (V) 100% EtOH (Merck) and 1/10 V 3 M Sodium Acetate pH 5.2 (Merck) and incubating the mixture at –20°C for at least 30 min. This was followed by centrifugation at 20 800 g; 4°C for 15 min (Eppendorf) and aspiration of the supernatant. The pellet was then washed with 500 µL cold 70% EtOH and again centrifuged at 20 800 g; 4°C for 5 min. Supernatant was removed and the pellet was left to air dry for about 15 min. The pellet was then resuspended in the desired volume of nuclease free water or TE buffer pH 8 (100 mM Tris; 10 mM EDTA).

2.17. Gel elution and DNA purification of PCR products

Both procedures were performed using the *Wizard SV Gel and PCR Clean-Up System* (Promega) according to the instructions of the manufacturer. Gel slices containing the bands of interest were cut from the gel with a scalpel and weighed. 1 µL *Membrane Binding Solution* was added per 1 mg of gel slice. The mixture was incubated at 60°C and occasionally vortexed until dissolved. PCR reaction was mixed directly with an equal amount of *Membrane Binding Solution*. The solution was then transferred to an *SV Minicolumn* which had been inserted into a collection tube and incubated at RT for 1 min. The assembly was centrifuged at 16 100 g (Eppendorf) and the flow through was discarded. 700 µL *Membrane Wash Solution* were added to the minicolumn and centrifugation was repeated for 1min. Flowthrough was discarded and another 500 µL wash solution were added, followed by centrifugation for 5 min. DNA was eluted by adding 30 µL of water to the minicolumn, incubating for 1 min and centrifugation into a fresh Eppendorf tube for 1 min.

2.18. DNA ligation

For the ligation of an insert into a vector backbone, 100 ng of vector backbone were mixed with 3 times and 5 times molar excess of insert and 5 to 10 Weiss units T4 DNA ligase (Fermentas). In most cases *Rapid Ligation Buffer* (Fermentas) was used and reaction was incubated for 15-30 min at room temperature (RT). It was later found that the use of regular ligation buffer and incubating the reaction at RT o/n seemingly yielded better results.

2.19. Preparation of competent *E. coli*

E. coli of the strains JM109 and XL1-Blue were made competent and stored for further use as described by Inoue et al. (Inoue, Nojima et al., 1990) with slight modifications. 5 mL SOB (20 g/L tryptone (Fluka); 5 g/L yeast extract (Oxoid); 0.5 g/L NaCl) were inoculated with either JM109 or XL1-Blue from a glycerol stock. The suspension was incubated on a shaker at 37°C o/n. The next day, 196 mL SOB

where mixed with 4 mL of the overnight culture and incubated on a shaker at 37°C until the OD_{600} had reached 0.55 (determined with *NanoDrop spectrophotometer*). Cells were then pelleted in a Sorvall RC5C centrifuge at 2 500 g and 4°C for 10 min. Supernatant was discarded completely and cells were resuspended in ice-cold *Inoue Transformation Buffer* (55 mM $MnCl_2.4H_2O$ (Merck); 15 mM $CaCl_2.4H_2O$ (Merck); 250 mM KCl (Merck), 10 mM PIPES (Fluka) (stock: 0.5 M; pH 6.7)). Cells were again harvested by centrifugation at 2 500 g 4°C for 10 min. Supernatant was removed completely and cells were resuspended in 20 mL of ice-cold Inoue buffer. 1.5 mL DMSO were added, the cell suspension was dispensed into 300 µL aliquots in pre-chilled 1.5 mL Eppendorf tubes and shock frozen with liquid nitrogen. Competent bacteria where then stored at -80°C until use.

2.20. Transformation of *E. Coli*

Competent *E. Coli* were thawed on ice. 100 µL bacterial cell suspension were mixed with 50% of ligation mix (typically 5–10 µL) and incubated on ice for 20 min. The bacteria where then heat-shocked at 42°C for 1-1.5 min. This was followed by addition of 1 mL of cold SOC medium (SOB; 20 mM glucose (Merck)) and incubation at 37°C; 350 rpm in a heat block for about 1 h. Finally, bacteria were plated on LB agar plates (20 g/L *LB Broth* (Sigma); 15 g/L agar (Fluka); petri dishes: Greiner) containing the appropriate antibiotic for selection (see Table 5) and incubated at 37°C o/n in a humidified incubator (Heraeus).

Table 5 Additives to media used for selection of bacteria

antibiotic	final concentration in growth medium
ampicillin (Roche)	50 µg/mL
kanamycin (Fluka)	50 µg/mL
gentamicin (Sigma)	7 µg/mL
X-gal (Fluka)	100 µg/mL
tetracycline (Sigma)	10 µg/mL
IPTG	40 µg/mL

2.21. Overnight culture and miniprep (boiling lysis)

To identify bacteria containing the desired plasmid, colonies were picked from LB agar plates and grown in 3 mL LB medium containing the appropriate antibiotic for selection (see Table 5) in vented tubes (Falcon) at 37°C; 200 rpm in a shaker (GFL) o/n. The next day, 1 mL cell suspension was transferred to an Eppendorf tube and cells were harvested at 16 100 g for 1 min. Supernatant was removed and cells were resuspended in 700 µL STET buffer (50 mM Tris; 50 mM EDTA; 20 g/L sucrose (USB); 5% Triton X-100). 13 µL lysozyme (10 mg/mL; Sigma) were added and the reaction was mixed and then heated to 100°C for 1.5 min. This was followed by centrifugation at 16 100 g for 10 min. The pellet was removed with a toothpick that had been tipped into RNAse A (10 mg/mL; Sigma) and 700 µL isopropanol (Merck) were added. The mixture was incubated at –20°C for 15 min and precipitated DNA was pelleted at 20 800 g for 15 min at 4°C. Supernatant was removed, the pellet was washed in 500 µL 70% EtOH and pelleted again at 20 800 g for 5 min at 4°C. Supernatant was completely removed and the pellet was dried at 37°C. It was then resuspended in 30 µL TE buffer.

2.22. Glycerol stocks

Glycerol stocks were created by mixing 700 µL overnight bacterial culture with 300 µL 80% glycerol. Stocks were stored at –80°C.

2.23. Miniprep

Plasmid minipreps were done using the *Wizard Plus SV Miniprep DNA Purification System* (Promega). 20 mL of bacterial overnight culture were centrifuged for 1 min at 15 000 g. Supernatant was removed and cells were resuspended in 250 µL *Cell Resuspension Solution*. 250 µL *Cell Lysis Solution* were added and the reaction was incubated for 3 min at RT. 10 µL *Alkaline Protease Solution* were then added and incubation was repeated for 5 min at RT. This step was succeeded by the adding of 350 µL *Neutralization Solution* and centrifugation at 16 100 g for 10 min. Supernatant was transferred to a *Spin Column* which had been inserted into a

Collection Tube and centrifuged for 1 min at 16 100 g. Flowthrough was discarded and 750 µL *Column Wash Solution* were added to the column. After centrifugation (1 min 16 100 g), flowthrough was discarded again and another 250 µL of fresh *Wash Solution* were added to the column and passed through by another step of centrifugation for 2 min. DNA was eluted by adding 50 µL of nuclease free water to the column, incubation for 2 min and centrifugation into an Eppendorf tube (16 100 g; 1 min).

2.24. Midiprep

Plasmid midipreps were performed using the *PureYield Plasmid Midiprep System* (Promega).

100 mL of bacterial overnight culture were harvested by centrifugation at 15 000 g for 1 min. The cell pellet was resuspended in 6 mL of *Cell Resuspension Solution* and lysed by addition of 6 mL *Cell Lysis Solution* followed by incubation at RT for 3 min. 10 mL *Neutralization Solution* were then added and the reaction was incubated for another 3 min. The lysate was pre-cleared by centrifugation at 15 000 g for 10 min. The supernatant was transferred to the *Clearing Column* which had been inserted into the *Binding Column*, attached to a vacuum manifold (Promega). Vacuum was applied (pump: Welch) until all liquid had passed through both columns. The *Clearing Column* was then discarded and 5 mL of *Endotoxin Removal Wash* were passed through the *Binding Column*. Next 20 mL of *Column Wash Solution* were passed through the column. Vacuum was continued for 30-60 sec following the pass through of all liquid. Remaining liquid was removed from the column by drying the drain with a paper towel. 600 µL water were used to elute DNA from the column by incubating it for 5 min and collecting the DNA solution with the help of an *Eluator Vacuum Elution Device* (Promega).

2.25. PCR

PCR reactions were carried out using either a *Robocycler Gradient 40* (Stratagene) or a *MyCycler* (with gradient upgrade; Bio-Rad) PCR machine. Analytical PCR was performed using *GoTaq DNA polymerase* (Promega) while for preparative PCR Pfu DNA Polymerase (Fermentas) was used. See Table 6 for reaction mixes. Primers were bought from Invitrogen and Eurogentec, see Table 10 for list of primers used.

Table 6 general reaction mixes for PCR

analytical PCR	**preparative PCR**
17.375 µL nuclease free water (Fermentas)	40.5 µL nuclease free water (Fermentas)
5 µL 5X Colorless GoTaq Reaction Buffer (Promega)	5 µL 10x Pfu buffer with $MgSO_4$ (Fermentas)
0.5 µL dNTPs (10mM; Fermentas)	1 µL dNTPs (10mM; Fermentas)
0.5 µL upstream primer (20 µM)	1 µL upstream primer (20 µM)
0.5 µL downstream primer (20 µM)	1 µL downstream primer (20 µM)
0.125 µL GoTaq DNA polymerase (5 units / µL)	0.5 µL Pfu DNA polymerase (2.5 units/µL)
1 µL template DNA	1 µL template DNA
when the Robocycler Gradient 40 PCR machine was used, 2 drops of mineral oil (Sigma) were put on top of the reaction mixes prior to the start of PCR	

2.26. Quantitative real-time PCR (QPCR)

Quantitative real-time PCR (QPCR) was used to determine the amount of viral DNA in supernatants of baculovirus infected Sf9 cells (see 3.3.1) and thereby determine the virus titer (Lo & Chao, 2004).

The Applied Biosystems *7500 Fast Real-Time PCR System* and the *Fast SYBR Green Master Mix* (Applied Biosystems; (Morrison, Weis et al., 1998; Schneeberger, Speiser et al., 1995) for a recent review see (Mackay, Arden et al., 2002)) were used to amplify and detect the PCR product.

For one QPCR reaction, 5 µL of isolated viral DNA (see 2.28) were mixed with 6 µL of 2 x *SYBR Green Master Mix* and 0.5 µL of each of the two primers (10 µM). For standard determination of Ct values for baculoviral sample DNA, primers *baculo_QPCR_for* (5'-CCCGTAACGGACCTCGTACTT-3') and *baculo_QPCR_rev* (5'-

TTATCGAGATTTATTTGCATACAACAAG-3') were used. Primer sequences were taken from (Lo & Chao, 2004).

The reaction was pipetted into 0.1 mL *MicroAmp Fast Optical 96-well Reaction Plates* (Applied Biosystems) and sealed with *MicroAmp Optical Adhesive Film* (Applied Biosystems). For each sample, QPCR was performed in duplicates, nuclease free water (Promega) was used as a negative control (no template control; NTC). QPCR was run in *Fast 7500 Mode* (see Table 7 for parameters), analysis was performed using *7500 Fast System SDS Software* (Applied Biosystems).

Table 7 cycling parameters for QPCR

thermal cycler profile				
stage	repetitions	temperature	time	ramp rate
1	1	95.0°C	00:30	Auto
2	45	95.0°C	00:03	Auto
		60.0°C	00:30	Auto

2.27. Generation of baculoviruses

The *Bac-To-Bac Baculovirus Expression System* (Invitrogen) was used to create recombinant baculoviruses for the expression of a gene of interest in both Sf9 insect cells (Luckow, 1993) and – with modifications - mammalian cells (Lackner, Genta et al., 2008).

The gene of interest was cloned into the multiple cloning site (MCS) of the *pFastBac* transfer plasmid containing the insect cell specific polyhedrin promoter (PPH; (Smith, Summers et al., 1983)). Alternatively, a modified version of the vector (*pFIP*) containing the cytomegalovirus immediate early gene promoter / enhancer (CMV; (Lackner, Genta et al., 2008)) was used. The purified plasmid (see 2.21) was then used to transform the *E. coli* strain *DH10 Bac* as described in 2.20 but with incubation at 37°C for 4-5 h before plating bacteria on LB agar plates containing kanamycin, tetracycline, gentamicin, X-gal and IPTG (see Table 5). Following incubation for 48 h at 37°C, large white colonies were subjected to PCR analysis to test for successful integration of the gene of interest into the bacmid. For this, a small amount of cells was transferred directly into the master mix (see 2.25) with a pipette tip. LB medium containing kanamycin and gentamicin was inoculated with

cells from colonies containing the gene of interest and incubated overnight at 37°C. The next day, the overnight cultures were used for the creation of glycerol stocks and miniprep of bacmids.

Bacteria were harvested from 1.5 mL of cell suspension by centrifugation (16 100 g; 1 min) and resuspended in 0.3 mL cold *solution 1* (15 mM Tris-HCl pH 8; 10 mM EDTA; 100 µg/mL RNase A). 0.3 mL *solution 2* (0.2 N NaOH; 1% SDS) were added and the reaction was incubated for 5 min at RT following gentle mixing. Next 0.3 mL of 3 M potassium acetate pH 5.5 were added and the mixture was placed on ice for 5-10 min. This was followed by centrifugation at 16 100 g for 10 min and transfer of the supernatant to an Eppendorf tube containing 0.8 mL isopropanol. The reaction was again placed on ice for 5-10 min and then centrifuged at RT for 15 min at 16 100 g. Supernatant was removed and the pellet was washed with 500 µL 70% EtOH. Following centrifugation at 16 100 g for 5 min, supernatant was removed and the pellet left to dry for a few minutes before being carefully resuspended (without pipetting or vortexing) in 40 µL TE pH 8. The bacmid DNA solution was stored at 4°C.

Sf9 cells growing in a T25 flask (5 mL medium) were then transfected with bacmid DNA as described under 2.5.

Five days to one week following transfection, cell supernatants were harvested from Sf9 cells and cleared by centrifugation at 1 000 g for 3 min. 50% of this P1 stock were then used to infect a T75 bottle (10 mL medium) almost confluent with Sf9 cells. Another week later supernatant was harvested in a similar manner as before and viral DNA isolated for determination of virus titer from this P2 stock. When necessary, amplification was continued until a virus stock with a suitable concentration of viral particles (1×10^7 - 1×10^9 Pfu[1]/mL) was reached. Virus stocks were stored at 4°C.

[1] plaque forming units

2.28. Isolation of baculoviral DNA from cell supernatants

Baculovirus DNA was isolated from Sf9 cell supernatant using the *High Pure Viral Nucleic Acid Kit* (Roche) according to the protocol suggested by the manufacturer.

In brief, 200 µL of cell supernatant (containing FBS), precleared by centrifugation at 1 000 g for 1 min, were mixed with 200 µL *Working Solution* (200 µL *Binding Solution* supplemented with 4 µL *carrier RNA*), freshly prepared for each experiment. 50 µL Proteinase K were added and the reaction was incubated at 72°C for 10 min following mixing. Next, 100 µL *Binding Solution* were added and the reaction was transferred to a *High Filter Tube*, inserted into a *Collection Tube*. The solution was passed through the column by centrifugation at 8 000 g for 1 min. Supernatant was discarded and 500 µL *Inhibitor Removal Buffer* were added to the column and passed through by centrifugation. Twice, 450 µL *Wash Buffer* were added to the column and passed through in a similar manner. Residual *Wash Buffer* was removed by centrifugation at maximum speed (16 100 g) for 1 min. The viral DNA was eluted into a 1.5 mL Eppendorf tube with 50 µL *Elution Buffer* and centrifugation at 8 000 g for 1 min.

2.29. Determination of virus titer

QPCR was used to determine the amount of viral particles in the medium / virus titer (Pfu/mL) as described by Lo et al. (Lo & Chao, 2004). A viral stock of known titer was used to correlate Ct-values to Pfu/mL (3.3).

Virus titer was determined from Ct-values of samples obtained by QPCR (see 2.26) with the following formulas:

Primer pair *baculo_QPCR_for* and *baculo_QPCR_rev*:

$$c = e^{-\left(\frac{Ct-35.294}{1.4332}\right)}$$

Primer pair *CMV_QPCR_for* and *CMV_QPCR_rev*:

$$c = e^{-\left(\frac{Ct-38.538}{1.6138}\right)}$$

c…virus titer in Pfu/mL

2.30. Infection of mammalian cells with baculovirus

Mammalian cells were infected by incubating them with a suitable amount of viral particles. Typically a multiplicity of infection (MOI) of 500-1000 (500 viral particles per mammalian cell) was used. The equivalent amount of virus stock was added directly to the medium of mammalian cells growing at about 75% confluency. About 24 h later, medium was removed, cells were washed several times with PBS and new growth medium was added. Gene expression was assessed 24-72 h post transfection.

2.31. Creation of conditioned medium

For the creation of medium containing secreted proteins, cells were grown to 75-100% confluency. Regular growth medium containing serum was removed from the cells and they were washed 2 x with PBS. In case of proteins artificially expressed in cells, this procedure was performed 24-48 h post transfection. A minimal amount of medium (2 mL in case of a T25 bottle) with or without serum was added and cells were incubated for 48 h. Medium was then harvested and cleared by centrifugation for 3 min at 1 500 rpm.

2.32. Precipitation of protein from cell supernatants and preparation for gel electrophoresis

Serum free cell supernatant was transferred to a SS34 centrifugation tube and 4 volumes of 100% EtOH were added. The solution was incubated at –20°C o/n. The next day precipitate was harvested by centrifugation at 4°C for 10 min at 15 000 g. Supernatant was removed, and the precipitate was washed in cold 70% EtOH and pelleted again at 16 100 g for 5 min. Supernatant was removed completely and the pellet was briefly left to dry. It was then resolubilized in a minimal volume (typically 25 µL for the precipitate from a T25 bottle) of *sample buffer* (7.5 M urea (Merck); 1.5 M thiourea (Sigma); 4% CHAPS (Merck); 0.05% SDS (Fluka)). Insoluble precipitate was removed by centrifugation at 16 100 g for 5 min. Finally 6 µL 5 x *SDS-PAGE loading buffer* (10% SDS; 60% glycerol; 300 mM Tris-HCl pH 6.8; 7% 2-mercaptoethanol (Aldrich); 0.025% bromophenolblue (Koch-Light Laboratories)) were added and the mixture was loaded onto the gel without prior heating to prevent carbamylation of the proteins in the sample (McCarthy, Hopwood et al., 2003).

2.33. Cell lysis and determination of protein concentration

To collect non-secreted protein from cells for SDS-PAGE and western blot analysis, around 500 000 cells growing in a 6-well were harvested by removing growth medium and scraping them (Cornig) into cold PBS. Cells were pelleted by centrifugation at 1 000 g for 2 min. All successive steps were done on ice. Supernatant was removed and cells were lysed by adding *Phospho-EGF-Receptor Lysis Buffer* (1 mM EDTA, 150 mM NaCl, 0.5 mM Na_3VO_4 (Sigma), 1.5 mM $MgCl_2$ (Fluka), 10% Glycerol, 50 mM HEPES (USB), 10 mM NaF (Sigma), 1% Triton X-100) and pipetting up and down as well as passing the cell through a thin syringe or sonification 3 x for 1 min (Bandelin). Insoluble components were then removed by centrifugation at 4°C and 20 800 g for 10 min. Supernatant was subjected to Bradford assay (*Bio-Rad Protein Assay*) to determine the protein concentration (Simonian & Smith, 2006). *Bio-Rad Protein Assay* was diluted 1:5 with

water and 199 µL of this solution for each of the samples to be measured and 6 x 198 µL for the creation of a standard curve were added to separate wells of a *PS microplate 96-well* (Greiner). Each of the wells for the standard curve received 1 µL of lysis buffer and an appropriate amount of a solution of BSA in water (0.1 µg/µL and 1 µg/µL) at different concentrations (see Figure 7 for an example of a standard curve). Each of the wells for the sample received 1 µL of cell lysate which had been diluted 1:3 in lysis buffer. Contents of the wells were mixed by pipetting and incubated at RT for 5 min. Absorption at 562 nm was determined using a *SynergyHT* plate reader and *Gen5* software (both BioTEK). The formula obtained from measurement of the standard curve was used to calculate the protein concentration in the samples.

	protein concentration of standard (µg/µL)	**abs at 562 nm**	**abs - abs(blank)**
blank	0	0.294	0.000
standard 1	0.1	0.322	0.028
standard 2	0.3	0.325	0.031
standard 3	0.5	0.377	0.083
standard 4	1	0.451	0.157
standard 5	2	0.554	0.260

abs…absorption

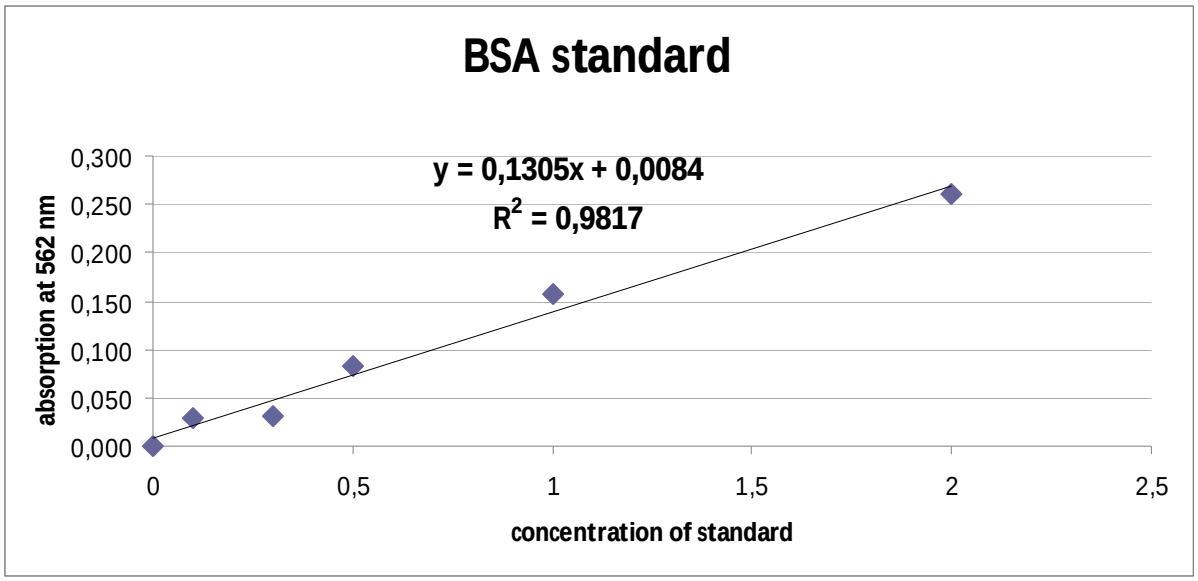


Figure 7 example of a standard curve for the determination of protein concentration by Bradford assay

2.34. Pulldown using anti-HA-agarose beads

To precipitate HA tagged protein from conditioned medium or cell lysate, a suitable amount (supernatant from at least one T25 bottle, cell lysate from at least one T75 bottle) was incubated with 50-100 µL (50% slurry) of *Anti-HA Affinity Matrix* (Roche) on a rotary shaker at 4°C o/n. The next day, beads were harvested by centrifugation at 1 500 g for 1 min and washed 5 x with 1 mL TBS 0.05% Tween 20; 0.1 mM EDTA. For elution of bound peptide, beads were incubated with 1 bed volume (BV) of HA peptide (Sigma) at 1 mg/mL in TBS 0.05% Tween 20; 0.1 mM EDTA for 15 min at 37°C. Alternatively, 100 mM glycine pH 2 was used in a similar manner or beads were boiled in 2 x SDS-PAGE loading buffer without reducing agents (e.g. 2-mercaptoethanol).

2.35. Polyacrylamide gel electrophoresis and western blot

For detection of a protein of interest in cell lysate or supernatant, samples were subjected to SDS-PAGE (Gallagher, 2007) and western blot (Gallagher, Winston et al., 2008). The *Mini Protean 3* system (Bio-Rad) was used for gel electrophoresis and transfer of protein to a PVDF membrane (*Hypond-P*; GE Healthcare) for immunodetection. Denaturing, discontinuous polyacrylamide gels with gel densities

of the separation between 7.5 and 15% were typically used. Occasionally *PAGEr Gold Precast Gels 4-20%* (Lonza) were used. The separation gel was cast and topped with isopropanol. Following polymerization (15-30 min), isopropanol was rinsed off with water which was removed with filter paper (Schleicher&Schuell). The stacking gel was then cast on top of the separating gel (for gel recipes see Table 8). Prior to loading, the gel pockets were cleaned with SDS running buffer (25 mM Tris; 192 mM glycine; 0.1% SDS) which was also used as running buffer for all SDS-PAGE gels. For each pocket 5-25 µg of protein lysate were mixed with 5 x SDS-PAGE loading buffer and heated for 5 min to 95°C before being loaded. In case of precipitated protein, the maximum volume possible per well was loaded (see 2.32). 5 µL of *Page Ruler Plus Prestained Protein Ladder* (Fermentas) were used as a marker. The gel was run at a voltage of 50 V for 30 min, followed by 110 V until the bromophenolblue tracking dye had left the gel.

Table 8 recipes for PAGE gels

		7.5%	**10%**	**12%**	**15%**		**stacking gel**
water	mL	2.379	1.960	1.625	1.122	Water	1.5
1.6 M Tris pH 8.8	mL	1.250	1.250	1.250	1.250	0.5 M Tris pH 6.8	0.625
10% SDS	µL	100	100	100	100	10% SDS	25
30% Acrylamid/Bis; 29:1 (Bio-Rad)	mL	1.256	1.675	2.010	2.513	30% Acrylamid/Bis; 29:1	325
10% APS (Merck)	µL	25	25	25	25	APS	25
TEMED (Amresco)	µL	5	5	5	5	TEMED	2.5

For Western blotting, separated proteins were transferred to a PVDF membrane using the tank transfer system (Gallagher, Winston et al., 2008). Before assembly of the blotting sandwich, the PVDF membrane was activated with methanol, rinsed thoroughly with tap water and equilibrated in *Towbin Buffer* (25 mM Tris; 192 mM Glycin; 5% MeOH) which was also used as transfer buffer. Transfer was performed at a current of 200 mA for 2 h at 4°C with a –20°C cooling pack in the tank.

Following transfer, the membrane was rinsed with distilled water and protein bands were visualized using Ponceau S staining. The membrane was incubated with *Ponceau S Solution* (0.5 g/L Ponceau S (Sigma); 1 mL/L glacial acetic acid (Gallagher, Winston et al., 2008)) for 5 min. Background staining was removed by

washing several times with distilled water. Staining was documented using a photocopier.

Following incubation, membranes were blocked with TBST-M (TBS: 8 g/L NaCl; 0.2 g/L KCl; 3 g/L Tris; pH 7.4; T: 0.1% Tween 20 (Sigma); M: 5% skim milk powder (Fluka)) for 1 h on a shaker at RT. Membranes were then washed 3 x 5 min with TBST and incubated in primary antibody solution overnight in a 15 mL Falcon tube on a shaker at 4°C. See Table 9 for antibodies used. The next day, membranes were washed 3 x with TBST and incubated in secondary antibody solution for 1 h on a shaker at RT. This was followed by washing 3 x with TBST and 2 x with TBS before incubation upside-down on a parafilm with *Immun-Star WesternC* reagent (Bio-Rad) and visualization of the signal with *Hyperfilm ECL* (GE Healthcare).

Table 9 antibodies used for western blotting

antibody	**dilution**	**supplier**	**diluent**	**size of target**
mouse-anti-Activin A	1:500	AbD Serotec; MCA950S	TBST-M	47/13kDa*
mouse-anti-GFP	1:2000	Roche; 11814460001	TBST-M	27kDa
mouse-anti-INHBE	1:500	Nigel Groome; (Vejda, Cranfield et al., 2002)	TBST-M	39/12kDa*
rabbit-anti-Akt	1:1000	Cell Signaling; 9272	TBST-5%BSA	60kDa
rabbit-anti-INHBE	1:500	Wolfgang Schneider; (Grusch, Drucker et al., 2006)	TBST-M	39/12kDa*
rabbit-anti-p44/42-MAPK	1:1000	Cell Signaling; 9102	TBST-5%BSA	44/42kDa
rabbit-anti-Phospho-Akt	1:1000	Cell Signaling; 9271	TBST-5%BSA	60kDa
rabbit-anti-Phospho-p44/42-MAPK	1:1000	Cell Signaling; 9101	TBST-5%BSA	44/42kDa
rabbit-anti-Phospho-S6	1:1000	Cell Signaling; 2211	TBST-5%BSA	32kDa
rabbit-anti-Phospho-Smad 1/5	1:1000	Cell Signaling; 9516	TBST-5%BSA	60kDa
rabbit-anti-Phospho-Smad 2	1:1000	Cell Signaling; 3101	TBST-M	60kDa
rabbit-anti-S6	1:1000	Cell Signaling; 2217	TBST-5%BSA	32kDa
rabbit-anti-Smad 2/3	1:1000	Cell Signaling; 3102	TBST-5%BSA	60kDa
rabbit-anti-Smad 5	1:1000	Cell Signaling; 9517	TBST-5%BSA	60kDa
rat-anti-HA	1:1000	Roche; 11867423001	TBST-M	n/a
goat-anti-rabbit-HRP	1:10000	Dako; P0448	TBST-M	n/a
goat-anti-rat-HRP	1:10000	Pierce; 31470	TBST-M	n/a
rabbit-anti-mouse-HRP	1:10000	Dako; P0260	TBST-M	n/a

rabbit-anti-rat-HRP	1:1000	Dako; P0450	TBST-M	n/a
*pro-protein/mature protein, monomeric				

2.36.SMAD phosphorylation assay

For determination of smad phosphorylation, HepG2 cells (smad 2) and HCT116 or HT1080 cells (smads 1 and 5) were seeded into 6-wells at about 500 000 cells per well in full medium. The next day, medium was removed and cells were washed 2 x with PBS. 0.5 mL of serum free medium conditioned media or purified recombinant cytokines were added, and the cells were incubated for 30 min in the incubator. For competition assays, wells were pre-incubated with 0.5 mL conditioned medium for 30 min in the incubator before the addition of activin A or BMP-2. Cells were then harvested as described under 2.33 and cell lysates were subjected to gel electrophoresis and western blot.

2.37.Microscopy

Light, fluorescence and fluorescence time-lapse microscopy were performed using an *Eclipse TE300* microscope (Nikon), a *Digital Sight DS-5M* (Nikon) or *SPOT INSIGHT* (Diagnostic Instruments) CCD camera and *MetaMorph* software (Molecular Devices). All microscopy was performed at room temperature.

3. Results

3.1. Construction of expression vectors coding for tagged and untagged versions of activins A and E

In order to investigate the effects of activin E as well as activin heterodimers containing the beta E subunit, an expression system was set up, as neither activin E nor any of the possible heterodimers are available commercially.

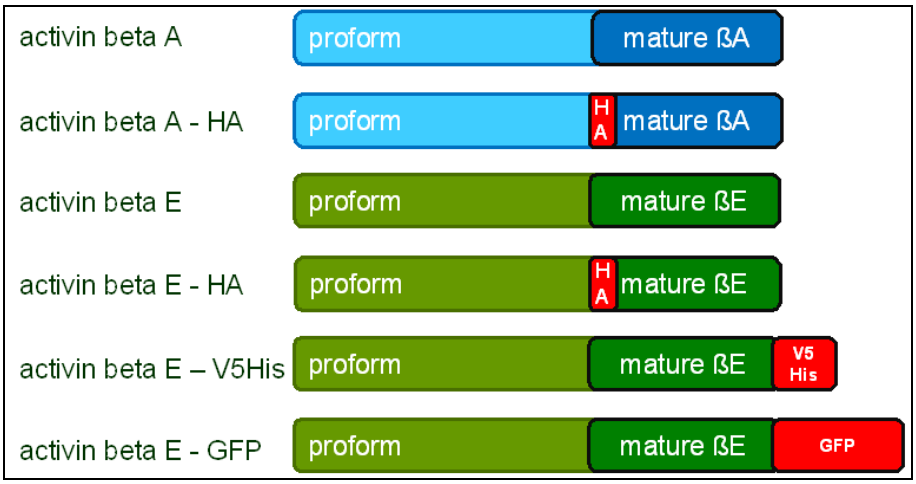
Since (i) purification of activins through classical biochemical means has been described as rather difficult (Smith, Price et al., 1990; Smith, Yaqoob et al., 1988) and because of (ii) reliable antibodies to activin beta E are not available, it was decided to create several tagged versions of the protein. Before my involvement in the project, the cDNA for human activin beta E had already been cloned into the multiple cloning site of a *pIRESneo3* vector (Clontech) for the expression in mammalian cells (*pIRES Hs AßE / D32*), by other members of the lab of Michael Grusch. In a similar manner, an expression vector for human activin beta A had been created (*pHs INHBA IRESpuro / D41*). In addition, a version of activin beta E containing the hemagglutinin (HA) sequence as a tag on the N-terminus of the mature form, right after the cleavage site of the prodomain had been produced (*pHsAßE-HA-IRESpuro / D46*).

This particular location for the tag had been chosen because of reports that a C-terminal tag rendered activin A inactive (Pangas & Woodruff, 2002), while a tag at the N-terminus of the prodomain would be cleaved off with the pro-peptide. A similar approach had been suggested and tested for activin A and chimeras of this protein by other groups (Fischer, Park et al., 2003). To confirm that this modification does not (necessarily) change the (unknown) biological activity of the protein, we also attempted to create an expression vector coding for a version of activin beta A, bearing the identical tag at the corresponding location (see 3.1.2).

Other approaches, like the expression in bacteria or the expression of the mature peptide only, had to be ruled out because the prodomain on the one hand renders the protein inactive if not cleaved off (Mason, Farnworth et al., 1996), but on the other hand is required for correct folding (Gray & Mason, 1990).

Since other reports suggested that variants of activin A containing a tag at the C-terminus would retain their biological activity (Munz, Smola et al., 1999) and that C-terminally tagged activin E would still be able to bind to follistatin (Hashimoto, Tsuchida et al., 2002), it was decided to also create several C-terminally tagged versions of activin beta E. In particular we created one version containing the V5-His tag (cloned into Invitrogen's *pcDNA3.1/V5-His* vector by members of the lab of Michael Grusch) at the C-terminus, and a chimerical protein in which activin beta E is fused to the N-terminus of enhanced green fluorescent protein (EGFP).

See Figure 8 for an overview of the different activin constructs created, and a list of the vectors on which they are encoded.



activin variant	**expression cassette found on vector**	**type of vector**
activin beta A	pHs INHBA IRESpuro / D41 INHBA_HA_IGP / G9	mam expr baculovirus (mam expr)
activin beta A – HA	*pINHBA_HA / D60 *INHBA_HA_IGP / G16	mam expr baculovirus (mam expr)
activin beta E	pIRES Hs AßE / D32 INHBE_IGP / G14	mam expr baculovirus (mam expr)
activin beta E – HA	pHsAßE-HA-IRESpuro / D46 INHBE_HA_IGP / G12 INHBE_HA_PPH / G19	mam expr baculovirus (mam expr) baculovirus (insect expr)
activin beta E – V5His	pcDNA-3-1-HsAßE-V5-HisB	mam expr
activin beta E – GFP	pINHBE_GFP / D57	mam expr
* later turned out to contain a frameshift resulting in a premature stop codon		

Figure 8 different variants of activin subunits and the vectors they are encoded on;
mam expr, mammalian cell expression vector
insect expr, insect cell expression vector

3.1.1. Construction of a mammalian expression vector coding for human activin beta E fused to EGFP (pINHBE_GFP / D57)

pINHBE_GFP was created by amplifying the cDNA sequence for activin beta E from *pIRES Hs AßE / D32* with primers removing the stop codon and containing a cleavage site for the restriction enzyme Bsp120I in the forward and reverse primer (*HsßE for around ATG*; *ßE removing stop codon*; see Table 10). PCR was performed using Pfu polymerase with an initial cycle of 3 min denaturation at 95°C, 50 sec annealing at 50°C and 4 min elongation at 72°C. This was followed by 37 cycles of 50 sec denaturation at 95°C, 50 sec annealing at 50°C and 2 min elongation at 72°C. The last cycle was performed with 50 sec denaturation at 95°C, 50 sec annealing at 50°C and 4 min elongation at 72°C. The PCR product was cleaned-up using the *Wizard SV Gel and PCR Clean-Up System* and digested with 10 units of Bsp120I (Fermentas) at 37°C for 3 h. The reaction product was run on a 2% gel and the band corresponding to the expected product of 1064 bp was excised and again cleaned-up using the *Wizard SV Gel and PCR Clean-Up System*. As a target vector, *pEGFP-N3* (Clontech) was digested with Bsp120I, dephosphorylated with CIAP and purified by being run on a gel and extracted with the *Wizard SV Gel and PCR Clean-Up System*.

Both fragments were then ligated using T4 DNA ligase and rapid ligation buffer and transformed into JM109 cells. Overnight cultures were created from several colonies, and those containing the correct plasmid were identified by digesting the purified plasmid (boiling lysis) with StuI and Eco147I (both Fermentas) see Figure 9.

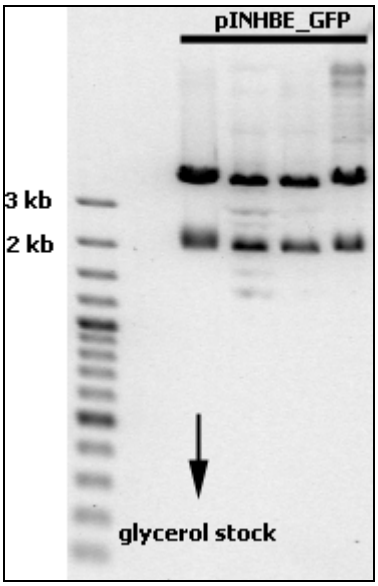


Figure 9 analytical digest of pINHBE_GFP;
Expected fragments: 3885 bp and 1938 bp

3.1.2. Construction of a mammalian expression vector coding for human activin beta A containing a HA-tag at the N-terminus of the mature form (pINHBA_HA / D60)

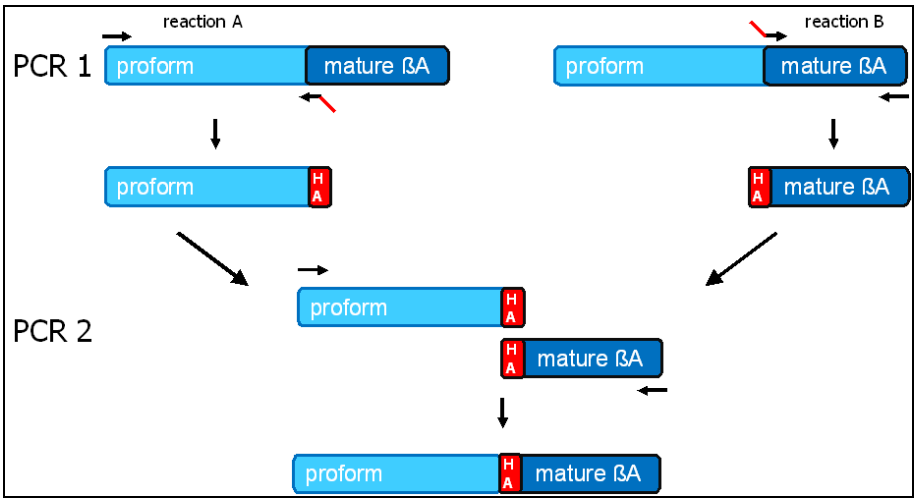


Figure 10 PCR strategy for the creation of pINHBA_HA;
PCR was used to add the HA-tag N-terminally of the proform and at the C-terminus of the mature peptide in two separate reactions (PCR 1, reaction A and B); the presence of the same sequence in both products allowed for an annealing of these two sequences in a second reaction – primers for the termini of the full length sequence were used to amplify the sequence for activin beta A – HA (PCR 2)

To insert a HA-tag C-terminally of the cleavage site in activin beta A, a PCR approach using four primers and two sequential amplifications was used (see Figure 10 for an outline of the strategy). In a first PCR (reaction A) one primer binding to the DNA

sequence coding for the N-terminus of activin beta A (*Hs Activin beta A for1*) and one binding to the sequence for C-terminus of the proform, but also containing the sequence for the HA-tag in addition (*pro-AßA-HA rev*) were used for the amplification. As a template, a plasmid containing the full length cDNA sequence of INHBA (*pHs INHBA IRESpuro / D41*) was used. Initial denaturing was 3 min at 95°C, followed by 40 cycles with 30 sec denaturing at 95°C, 30 sec annealing at 61.5°C and 2 min elongation at 72°C, and a final elongation for 4 min at 72°C. In a separate reaction (reaction B) with the same conditions, a primer containing the HA-tag sequence and binding to the N-terminus of the mature form (*HA-Tag-AßA-mat*), and one primer binding to the C-terminus of INHBA (*Hs Activin beta A rev1*) were used with the same template as before. The two products were subjected to gel electrophoresis. The DNA fragments running at the expected heights (see Figure 11, left) were excised and purified with the *Wizard SV Gel and PCR Clean-Up System*. For a second PCR reaction, the products from the first set of reactions were mixed (1/100 of each of the total purified products) and amplified using the two primers binding to the N- and C-termini of full length INHBA (*Hs Activin beta A for1* and *Hs Activin beta A rev1*). Initial denaturing was 3 min at 95°C, followed by 40 cycles with 30 sec denaturing at 95°C, 30 sec annealing at 57°C and 3 min elongation at 72°C, and final elongation for 4 min at 72°C. The product was again subjected to gel electrophoresis (see Figure 11, right), purified, phosphorylated with PNK and ligated into the vector *pIRESneo3* which had been digested with EcoRV (Fermentas) and dephosphorylated. Following transfection into JM109 cells, overnight culture and boiling lysis miniprep, the identity of the plasmid was confirmed by digest with BamHI (Fermentas) yielding the expected fragments of 1312 bp and 5274 bp (not shown).

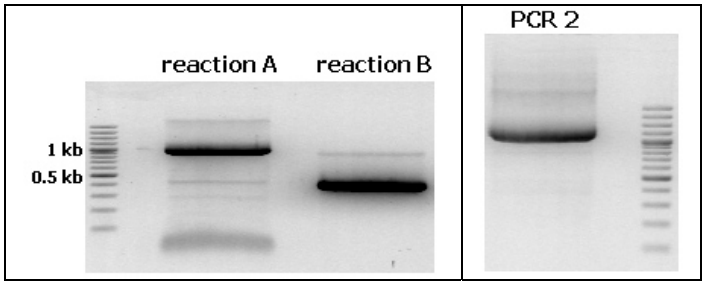


Figure 11 products of the first set of PCR reactions for the creation of pINHBA_HA;
sizes of product are as expected

Unfortunately, sequencing of the vector later showed that a error had occurred during the creation of the fragment. It resulted in a frameshift and premature stop codon about 200 bases from the 3' end of the expected open reading frame. Despite thorough re-evaluation of the strategy, the exact reason for this remained unknown. As a repetition of the PCR yielded the correct product, an error of the polymerase during the initial amplification procedure is the most likely explanation.

Table 10 PCR primers used

primer Name	sequence 5' to 3'	internal designation
HsßE for around ATG	TATTAGGGCCCAGGAGCATGCGGCTCCCTGA	83
baculo_QPCR_for	CCCGTAACGGACCTCGTACTT	201
baculo_QPCR_rev	TTATCGAGATTTATTTGCATACAACAAG	202
CMV_QPCR_for	CCCATAGTAACGCCAATAGG	203
CMV_QPCR_rev	CGTAGATGTACTGCCAAGTAGG	204
gent_rev	ATCAGCCGGACTCCGATTACC	205
GFP-rev	CTGAACTTGTGGCCGTTTAC	41
HA-Tag-AßA-mat	TACCCATACGATGTTCCAGATTACGCTTTGGAGTGTGATGGCAAGG	190
Hs Activin beta A for1_EcoRI	CGTGAATTCGGATGCCCTTGCTTTGG	197
Hs Activin beta E rev1	CCCAGTTCCTGGAAGTCTAC	6B
Hs Activin beta A for1	CAGGATGCCCTTGCTTTGG	1A
Hs Activin beta A rev1	TGGGCTGGGCAACTCTATG	1B
IRES rev2	TTATCATCGTGTTTTTCAAAGG	18B
M13 for (-40) Invitrogen	GTTTTCCCAGTCACGAC	21A
pro-AßA-HA rev	AGCGTAATCTGGAACATCGTATGGGTAGCCCCGCCGACGCCGGCGAT	189
ßE removing stop codon	TATTAGGGCCCATGCTGCAGCCACAGGCCTCCA	85

3.1.3. Prediction of furin cleavage sites in different activin variants

To get a hint whether or not the addition of epitope tags (in particular close to the cleavage site of the protein) would have an effect on the processing of the activin subunits, the sequence of the expected full length protein was evaluated using a publicly available neuronal network for the prediction of furin cleavage sites (Duckert, Brunak et al., 2004). For results see Figure 12.

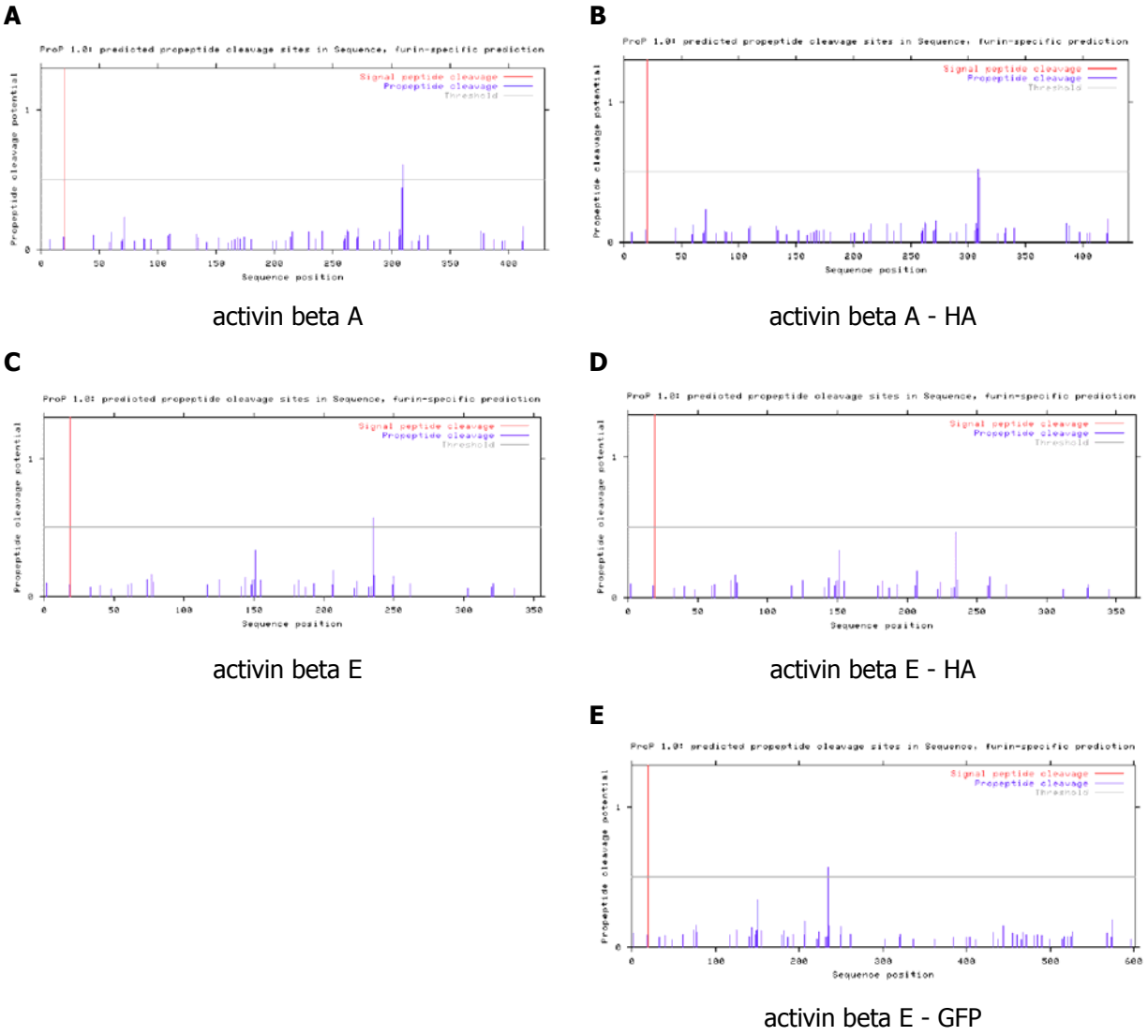


Figure 12 graphical representation of prediction of the site of cleavage by furin; protein sequences of wildtype activin subunits beta A and beta E (A, C) as well as tagged activin subunits beta A – HA (B), beta E – HA (D) and beta E – GFP (E) were analyzed for the presence of furin cleavage sites (x-axis: position in the amino acid sequence of the polypeptide, y-axis: relative likelihood of cleavage by furin); predicted cleavage sites for wildtype activins are in accordance with published data, the presence of tags does not have a major effect on cleavage in the prediction

An evaluation of the suggested cleavage sites in the wildtype forms of activin beta A and activin beta E (*D41* and *D32* respectively) shows that they coincide with the cleavage sites reported (Hashimoto, Tsuchida et al., 2002; Mason, Farnworth et al., 1996) and our own observations for activin A (see below).

The HA-tag is predicted to slightly reduce the effectiveness of the cleavage site, while the C-terminal fusion to GFP should not affect processing.

3.2. Stable cells lines

In order to have continuous access to recombinantly produced activins without the need to transfect cells and verify the expression of the protein for every experiment, CHO-DHFR$^-$ and HEK293 cells were transfected with vectors coding for various variants of different activins and selected for stable integration of the plasmid into their genome. As described in the materials and methods section, CHO-DHFR$^-$ cells were cotransfected with a plasmid expressing DHFR to increase their expression of the gene of interest by treatment with methotrexate once stable clones had been selected. See Table 11 for a list of stable cell lines that were created during the course of the diploma thesis.

CHO cells were preferentially used for the creation of stable clones, as they proved to be fast growing and very resistant to unfavorable conditions like the incubation with serum-free medium. They have often been described as ideal for the expression of proteins (Kingston, Kaufman et al., 2002), and have been used successfully for the production of activin A in the past (Arai, Tsuchida et al., 2006).

Table 11 cell lines created

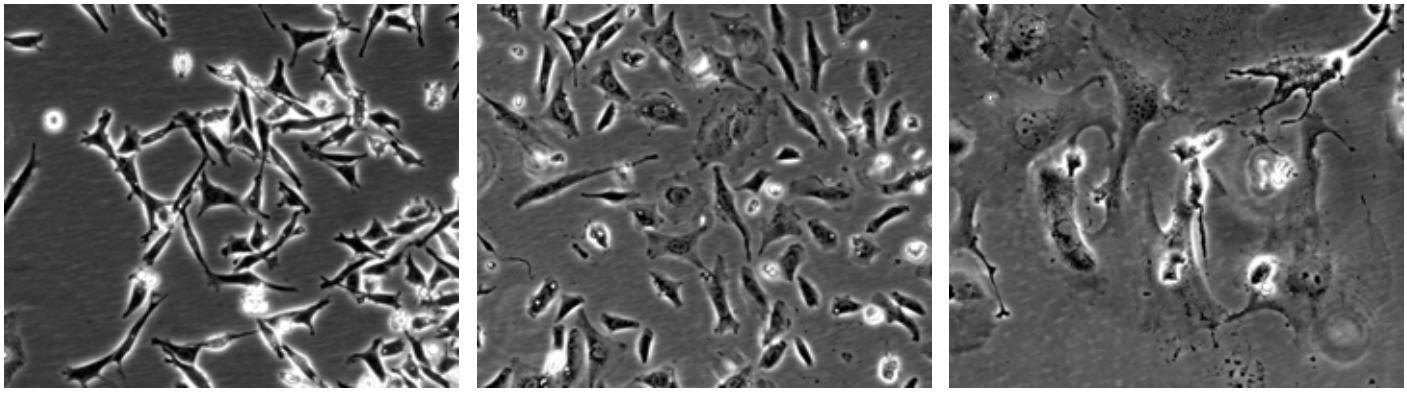
cell line	plasmid used for transfection	MTX	transgene to be expressed / function
*293-D43	pcDNA-3-1-HsAßE-V5-HisB		activin beta E - V5His
293-D48	pACVR2B-ECFP		cctivin receptor 2B - ECFP
293-D49	BMPR1B-EYFP		BMP receptor 1B - EYFP
CHO-A69	pECFP-IRESpuro		ECFP / control
CHO-A71	pIRES-puro		puromycin resistance marker / control
CHO-A8	pcDNA3		neomycin resistance marker / control
CHO-D32	pIRES Hs AßE	80 nM	activin beta E
*CHO-D43	pcDNA-3-1-HsAßE-V5-HisB		activin beta E - V5His
CHO-D46	pHsAßE-HA-IRESpuro	320 nM	activin beta E - HA
CHO-D57	pINHBE_GFP	5 uM	activin beta E - GFP
**CHO-D60	pINHBA_HA		activin beta A - HA

* the use of the cell line was stopped as western blotting gave no evidence for the expression of the transgene; CHO-D43 cells in addition showed significantly faster growth than cell lines expressing other versions of INHBE and had a morphology that resembled CHO cells transfected with the resistance gene only

** D60 turned out to contain an error in the sequence (see 3.1.2)

Unfortunately, several attempts to create a cell line stably expressing the wildtype form of activin A failed. – Stable clones appeared and grew to about 25% confluence, but 75-90% always died upon reseeding, making expansion impossible.

Apart from the determination of protein expression by immunological techniques (see below), established cell lines were investigated for their growth characteristics (3.5.1) and their morphology. It soon became evident, that expression of both wildtype and HA-tagged variants of activin beta E resulted in a much more spread out morphology of CHO cells in comparison to cells just expressing the resistance markers to the respective antibiotics used for selection (see Figure 13).



CHO-A71 CHO-D32 CHO-D46

Figure 13 phase contrast images of CHO cells stably expressing variants of activin beta E or resistance genes only; all images were taken at the same magnification;
cells of cell lines CHO-D32, expressing activin beta E, and in particular CHO-D46, expressing activin beta E – HA, have significantly distinct morphologies than CHO-A71 cells, expressing the puromycin resistance marker only

3.3. Creation of a baculovirus expression system for activins

In order to be able to produce large amounts of recombinant activin variants in different cell lines, we established a baculoviral expression system for different activins.

Based on the *Bac-To-Bac Baculovirus Expression System* (Invitrogen), the group of Michael Grusch had previously adapted the system for efficient expression of genes in mammalian cells (Lackner, Genta et al., 2008). In brief, the system makes use of transfer vectors (in the original system called *Fastbacs*), containing a CMV promoter, a multiple cloning site (MCS) to insert the gene of interest (GOI), and a fusion

protein between EGFP and a puromycin resistance protein, encoded on the same bicistronic mRNA, separated by an IRES sequence. Baculoviral systems like this, containing a mammalian expression cassette, are generally referred to as *BacMams* (Fornwald, Lu et al., 2007).

The expression cassette was transferred into the virus genome (bacmid) via recombination, taking place in the *E. coli* strain DH10 containing the bacmid and a recombinase. See Figure 14 for a general outline. Upon successful recombination via Tn7 sites, the gene coding for the lacZalpha protein fragment was destroyed, resulting in a loss of blue staining of the respective colonies when plated on X-Gal / IPTG containing agar plates. White colonies were checked for the presence of the integration cassette using PCR and used for the inoculation of overnight cultures. Bacmid DNA was isolated from these cultures and used to transfect Sf9 insect cells which then produce the virus. When cell supernatant containing a sufficiently high virus titer (see below) had been created by re-infecting Sf9 cells with cell supernatant from previously infected cells, the supernatants were directly used to infect mammalian cells. Successful infection was determined by green fluorescence resulting from the expression of the GFP-containing fusion protein from the same mRNA as the gene of interest (see Figure 22).

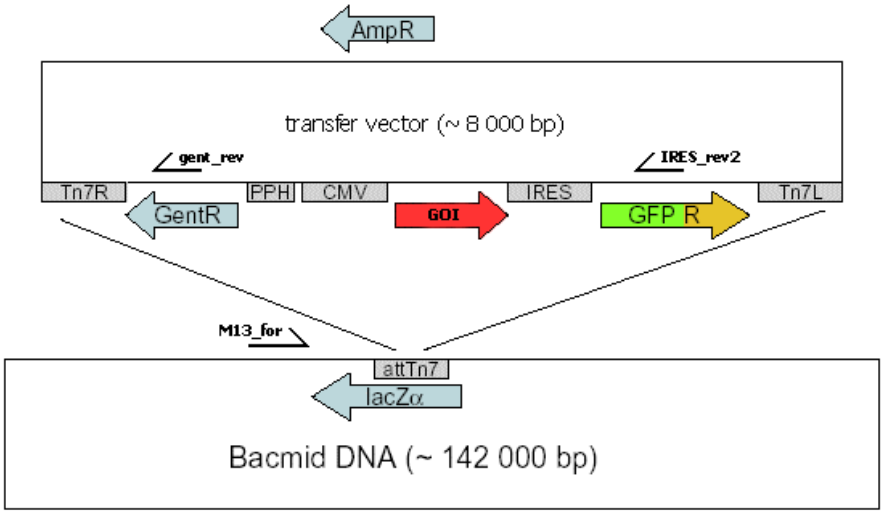


Figure 14 transfer vector and bacmid
the transfer vector containing the expression cassette is created by standard cloning procedures; integration into the virus genome (bacmid) takes place via Tn7 sites in DH10 *E. coli* cells; successful recombination is assessed by PCR using primers binding on the original bacmid and the region donated by the transfer vector, respectively

3.3.1. Optimization of procedures for production and quantification of baculoviruses

During the construction of new baculoviruses (see below), we were able to optimize the production of baculoviruses and determination of the virus titer, both in respect of financial resources and hands on time required.

In particular, we established the use of plasmids obtained by boiling lysis miniprep for the transformation of DH10 cells and the use of the primer pair *M13 for (-40) Invitrogen* and *gent_rev* as opposed to *M13 for (-40) Invitrogen* and *IRES rev2* (see Table 10) directly on white colonies (colony PCR) instead of on purified plasmids (initial denaturing 3 min, 95°C; 33 cycles with 30 sec denaturing, 95°C, 30 sec annealing 60°C, 1 min elongation, 72°C, final elongation 2 min, 72°C). This on the one hand made it possible to immediately proceed to the next step with the miniprep used for checking of the transfer vector, by this saving one day in the production of a virus. On the other hand, the PCR modification gave rise to a much smaller product (800 bp instead of 4 200 bp), significantly reducing the time for the PCR and making it unnecessary to isolate bacmid DNA from several possible candidates.

Much more significant however was the establishment of QPCR for the determination of baculovirus titer. While the previously used *BacPAK Baculovirus Rapid Titer Kit* (Clontech) (Kitts & Green, 1999) produced good results, it was both time consuming and expensive. Therefore, we switched to the use of QPCR as suggested by Lo et al. (Lo & Chao, 2004). In addition to the primers *baculo_QPCR_for* and *baculo_QPCR_rev*, described in their study, we designed the primers *CMV_QPCR_for* and *CMV_QPCR_rev*, binding inside the CMV promotor, to be able to quantify viruses containing the CMV expression cassette only. For both primer pairs, we determined a formula to calculate the virus titer in Pfu/mL from the Ct-values obtained by QPCR of different dilutions of a baculovirus with known titer (previously determined by *BacPAK Baculovirus Rapid Titer Kit*). For standard curves see Figure 15, for formulas see 2.29. Typical virus titers were in the range of 3×10^6 - 3×10^8 Pfu/mL. In general, results from the determination of the virus titer of one sample with the two different primer pairs were in good agreement.

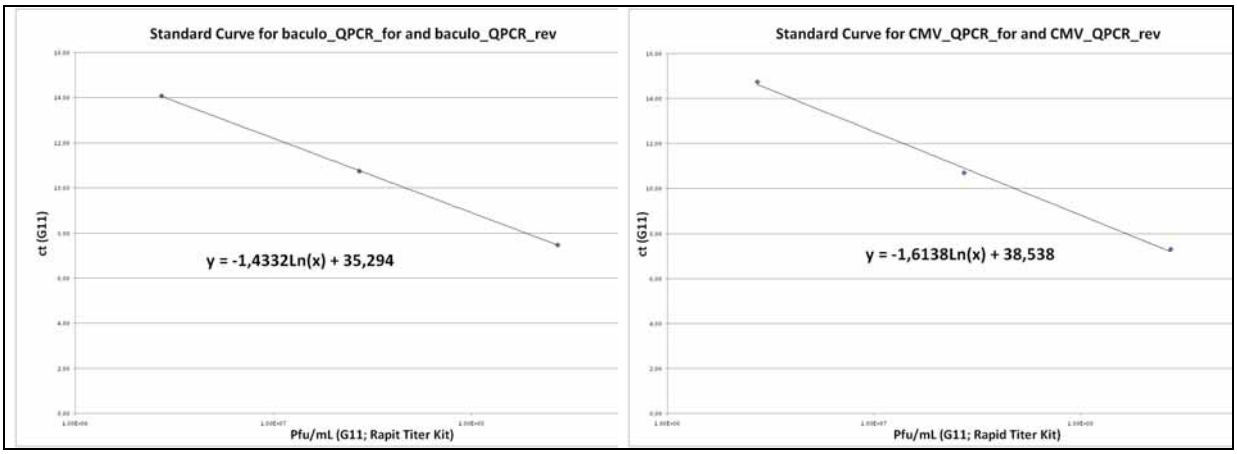


Figure 15 standard curves for determination of the baculotiter from Ct-values obtained by QPCR;
curves were created by determining the Ct-values of different dilutions of a baculovirus with known titer

3.3.2. Construction of a baculovirus for the expression of HA-tagged activin beta A (INHBA_HA_IGP / G16)

The sequence coding for HA-tagged activin A was cloned into the transfer vector *pFastbac IRES EGFPpuro / A82* by PCR amplification from the *D60* vector (see 3.1.2) with the primers *Hs Activin beta A for1_EcoRI* and *Hs Activin beta A rev1* (initial denaturing 3 min 95°C, 40 cycles of 30 sec 95°C, 50 sec 55°C and 3 min 72°C; final elongation 4 min 72°C). After phosphorylation of the sequence of interest, it was inserted into the vector *A82* which had been cut with Eco47III (Fermentas) and dephosphorylated with CIAP. Plasmids from the resulting colonies were checked for their identity by digest with PstI (Fermentas) and used to transform DH10 cells. Integration into the bacmids of white colonies was checked with colony PCR with the primers *M13 for (-40) Invitrogen* and *IRES rev2* (initial denaturing 5 min 95°C, 33 cycles of 50 sec 95°C, 50 sec 60°C and 8 min 72°C; final elongation 2 min 72°C). Sf9 cells were transfected with the purified bacmid and isolated virus again checked for its identity with primers *Hs Activin beta A for1* and *GFP-rev* (initial denaturing 2 min 95°C, 33 cycles of 30 sec 95°C, 30 sec 55°C and 3.5 min 72°C; final elongation 5 min 72°C).

Like the template vector *D60* that had given rise to the fragment inserted into the transfer vector, the resulting baculovirus must unfortunately also contain a mutation

in the sequence coding for activin beta A – HA, resulting in a frameshift in the mature part (see 3.1.2).

3.3.3. Construction of a baculovirus for the expression of wildtype activin beta E (INHBE_IGP / G14)

While a baculovirus containing an expression cassette for HA-tagged activin beta E and wildtype activin beta A had already been created by other members of the group of Michael Grusch, we decided to create an expression vector for wildtype activin beta E. To do so, we digested the vector *D32*, containing the cDNA for full length human activin beta E with NdeI and HpaI and cloned it into the transfer vector *A82* which had been cut with NdeI and Eco47III and dephosphorylated with CIAP (all: Fermentas). After confirmation of the identity of the vector by digest with Eco47III, it was transformed into DH10 cells. Integration of the insert was confirmed as outlined for *G16* (see 3.3.2). Identity of the virus produced by infected Sf9 cells was checked by PCR with primers *M13 for (-40) Invitrogen* and *Hs Activin beta E rev1* (same conditions as for final check of G16 – see 3.3.2).

3.3.4. Construction of a baculovirus for the expression of HA-tagged activin beta E in insect cells (INHBE_HA_PPH / G19)

As insect cells had previously been used successfully for the production of activins (Wuytens, Verschueren et al., 1999), we decided to test the same approach for the production of HA-tagged activin beta E. The transfer vector was created by cloning the INHBE-HA containing fragment of the vector *D46* (3.1) into the MCS of *pFastbac-1 / A93*, by digesting both with HindIII (Fermentas). Identity was confirmed by digest with SnaBI (New England Biolabs). Colonies of DH10 cells in which the expected bacmid had been created by recombination were identified by PCR with primers *M13 for (-40) Invitrogen* and *gent_rev* as outlined under 3.3.1. Finally, production of a virus by Sf9 cells containing the correct expression cassette was checked by PCR with primers *M13 for (-40) Invitrogen* and *Hs Activin beta E rev1* (same conditions as for final check of G14 – see 3.3.3).

3.4. Determination of expression of wild type and tagged versions of Activin A and E

3.4.1. Different precipitation techniques

To determine the ideal method to precipitate activins from serum free cell supernatant, different approaches as proposed by Zellner et al. (Zellner, Winkler et al., 2005) and Jiang et al. (Jiang, He et al., 2004) were tested. In addition to protein precipitation with EtOH (addition of 4 volumes (V) of 100% EtOH; incubation at –20°C o/n) which was used as the standard method, acetone (addition of 4 V of acetone; –20°C o/n), trichloroacetic acid (TCA, Merck; 1/3 V of 6.1 N TCA; 1 h 4°C), ammonium sulfate (AMS, Merck; addition of 9 V saturated AMS solution; mixing on ice for 30 min) and chloroform-methanol (addition of 4 V MeOH; 1 V chloroform (Merck); 3 V H_2O; 1 min 15 000 g; removal of upper phase; 4 V (original volume) MeOH; 2 min 15 000 g; removal of supernatant) were used to precipitate protein from conditioned media. In all cases except for chloroform-methanol, precipitation was completed by centrifugation at 15 000 g at 4°C for 15 min and removal of the supernatant. All pellets were resuspended in 1 x SDS-PAGE loading buffer, neutralized where necessary and heated to 95°C before being loaded onto the gel.

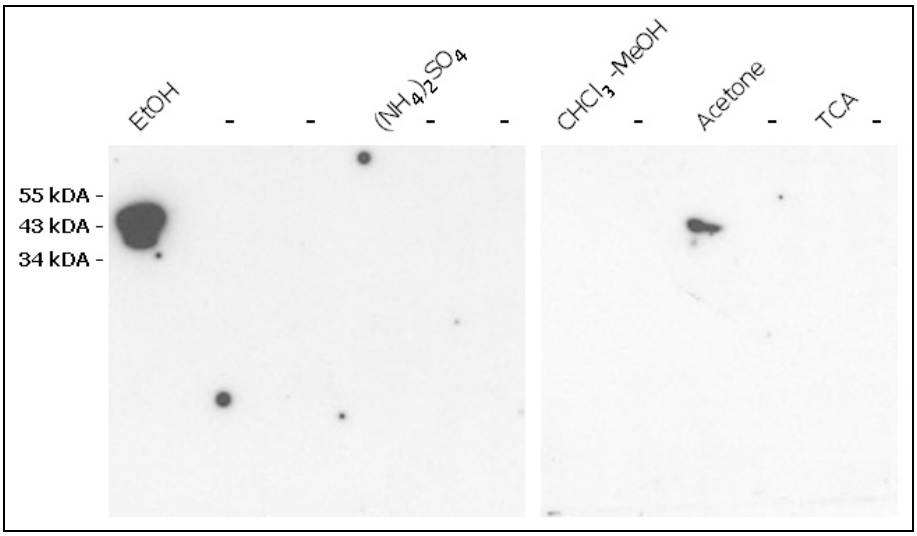


Figure 16 comparison of different methods for the precipitation of HA-tagged activin beta E from cell supernatant;
conditioned medium (5 mL per lane) from a confluent T25 flask of CHO cells expressing activin beta E – HA was precipitated as described in the text; the membranes were probed with antibody against the HA-tag

In Figure 16 it can clearly be seen that precipitation with ethanol yielded the best results. While there is still a band at the correct height visible in case of precipitation with acetone, all other precipitation methods failed to produce the expected results. Although optimizing the different procedures might lead to more similar results, we decided to use EtOH as the standard means of isolating protein from cell supernatant as it is also straight forward, reliable and cost-effective.

3.4.2. Western blot

Western blots were the main way of determining the expression of the gene of interest. In a first line of experiments, it was tested whether or not it was possible to isolate and visualize different activins overexpressed in mammalian cells. To do so, HEK293 cells were transfected with different plasmids coding for members of the activin family. Among them was *pHs INHBA IRESpuro / D41*. Protein from conditioned media from cells transfected with the construct was precipitated and run on a SDS-PAGE gel, next to purified recombinant activin A (R&D) and, following western blotting, analyzed by immunodetection with mouse-anti-Activin A antibody (see Figure 17).

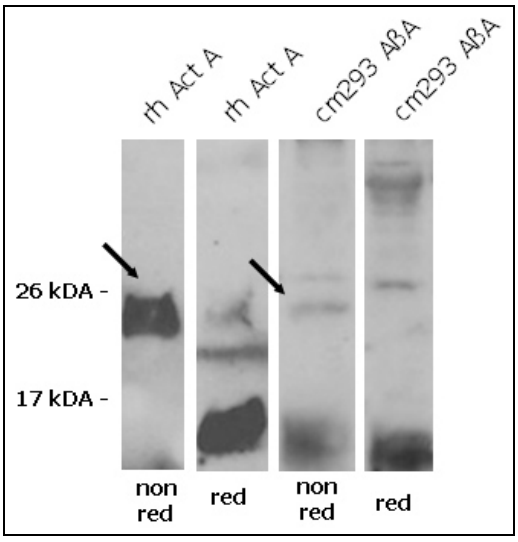


Figure 17 recombinant and exogenously expressed activin A under reducing and non-reducing conditions;
purified recombinant activin A (rh Act A) and precipitate of conditioned media of HEK293 cells (one T25 flask), transiently expressing activin beta A (cm293 AβA) were run on a gel under reducing and non-reducing conditions; the membrane was probed with antibodies against activin A; the mature dimeric form of activin beta A (activin A) is marked by black arrows

In accordance with the results described by Smith et al. (Smith, Price et al., 1990), we detected a band for the mature dimeric activin A (marked by an arrow) under non-reducing conditions at a position a little below 26 kDa, and a band for mature monomeric activin beta A under reducing conditions below 17 kDa. While the band corresponding to the monomeric version can be found in the supernatants of HEK293 cells run both under reducing and non-reducing conditions, the band corresponding to the mature dimer (arrows) is only present under non-reducing conditions. This indicates that activin A can be expressed in, and is processed correctly by HEK293 cells.

The same approach was used to determine the expression of human activin A in HepG2 cells that had been infected with the *G9* baculovirus, containing an expression cassette for INHBA.

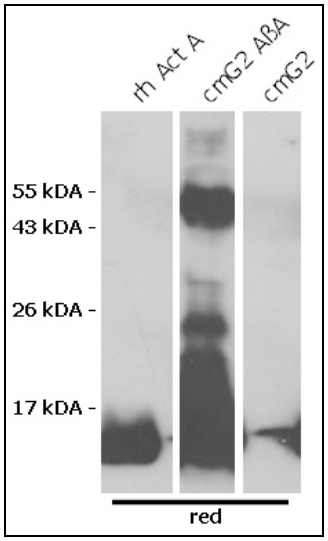


Figure 18 supernatants from HepG2 cells infected with baculovirus for activin beta A expression (G9);
protein was precipitated from conditioned media from HepG2 cells growing in 6-wells, either untreated (cmG2) or transduced with a baculovirus containing an expression cassette for activin beta A (cmG2 AβA); recombinant activin A (10 ng) was used as a control

It can be seen from Figure 18, that infection of HepG2 cells with G9 baculovirus, containing an expression cassette for activin beta A results in high level expression of the protein of interest. The bands appear specific, as they are almost non-existent in the cell supernatant of uninfected HepG2 cells. The minor band in the HepG2 lane most likely results from spill over during the loading of the gel as HepG2 cells normally do not express the INHBA gene (Chen, Woodruff et al., 2000). The band

found in supernatant of HepG2 cells transduced with the G9 virus between 43 kDa and 55 kDa most likely corresponds to the monomeric proform of activin A (47 kDa without glycosylation) which was also found by other researchers (Arai, Tsuchida et al., 2006).

Due to the low affinity (50 ng but not 10 ng of recombinant mature peptide of activin beta E (Biopharm) could be detected with mouse-anti-INHBE antibody; see Figure 19; left) and high cross-reactivity of all antibodies raised against activin beta E (data not shown) that we had access to, we were not able to visualize the wildtype form of the protein artificially expressed from *pIRES Hs AßE / D32* in different cell systems. Because of this, it was never possible to prove the expression of wildtype activin E, and we had to use RNA expression (not shown) and other criteria like the morphology and growth characteristics of stable cell lines (see 3.2 and 3.5.1) in comparison to those expressing tagged versions of the protein to judge the expression of the protein.
For that reason, the cell line CHO-D46, stably expressing the HA-tagged version of activin beta E, was established and used for many of the experiments trying to determine the biological function of activin beta E.
In addition, the fusion protein between activin beta E and GFP, expressed by the CHO-D57 cell line (see also Figure 22), was used for detection of the secreted protein and several of the experiments.

Figure 19 shows western blots of recombinant mature human (rh) activin beta E, expressed in *E. coli*, detected with mouse-anti-INHBE antibody, and protein from supernatant from CHO-D46 cells as well as cells infected with the *G12* baculovirus containing the same expression cassette (both detected with an antibody against the HA epitope).

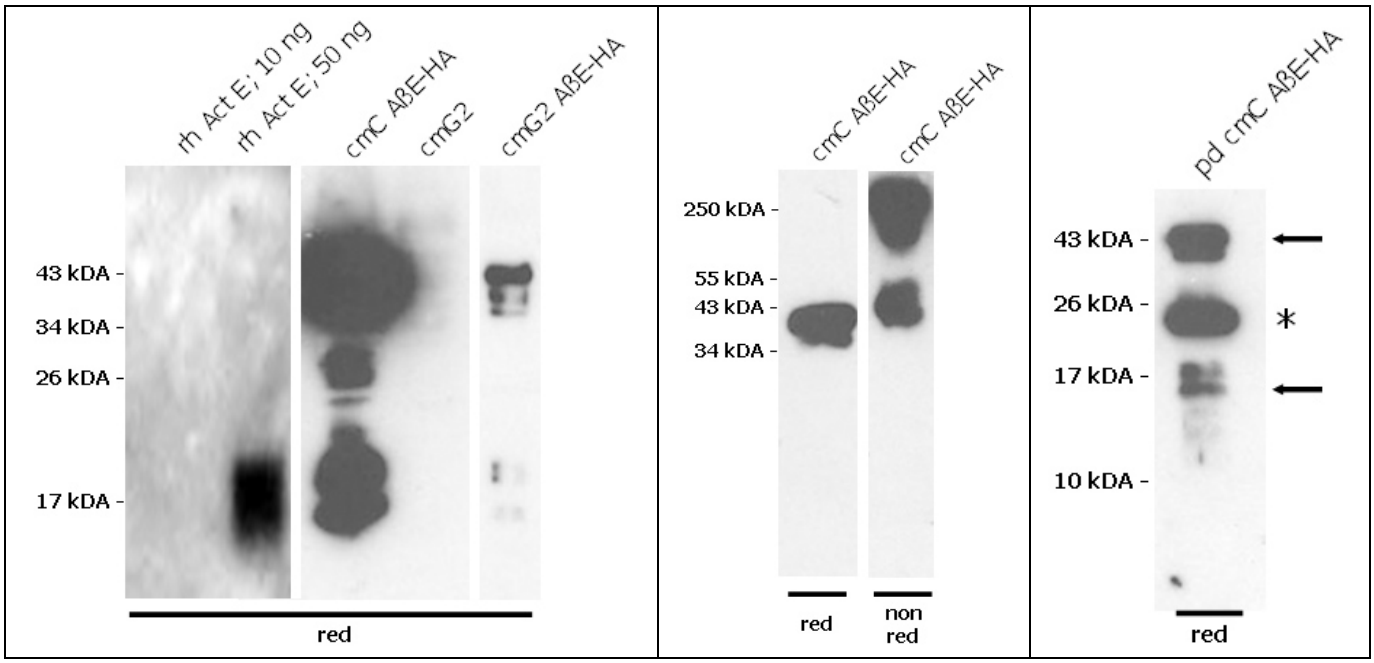


Figure 19 western blot of recombinant activin beta E and HA-tagged activin beta E produced by the CHO-D46 cell line and HepG2 cells infected with the baculovirus for activin beta E-HA expression (left);
eluate from anti-HA-agarose pulldown of activin beta E under reducing and non-reducing conditions (middle);
pulldown of activin beta E – HA containing supernatants (right);
conditioned media of CHO cells expressing activin beta E – HA (cmC AßE-HA) or of HepG2 cells either untreated (cmG2) or transduced with the G12 baculovirus containing an expression cassette for activin beta E – HA (cmG2 AßE-HA) were subjected to gel electrophoresis and western blot under reducing conditions – pro-protein and the mature form of activin beta E could be detected (left); under non-reducing conditions, large aggregates were detected (middle);
pulldown of similar CHO supernatants using anti-HA-agarose (pd cmC AßE-HA), followed by western blot under reducing conditions yielded bands for the pro-protein and the mature protein (marked by arrows), as well as a signal most likely caused by eluted antibody (asterisk) (right); see text for additional information

Antibodies directed against the wildtype protein detect a clear band below 17 kDa when (large amounts of) recombinant activin beta E are loaded onto the gel. A band similar in height can also be seen in the supernatant of CHO-D46 cells and HepG2 cells expressing the same construct after viral transduction, but not in case of untransfected HepG2 cells. It is also in accordance with a band for monomeric mature activin beta E (expected: 13.5 kDa) described in a mouse overexpressing this protein (Hashimoto, Ushiro et al., 2006). A much more intense band however, which is also described in the same publication, is detected between 43 kDa and 34 kDa. In all likelihood it is caused by the proform (expected to be 39.6 kDa without glycosylation). Closer evaluation shows that it is indeed a double band (compare to Figure 16), probably caused by versions of the protein with a different glycosylation

status (Vejda, Cranfield et al., 2002). It has to be noted, that while a band around 43 kDa could be detected in almost every precipitate from the supernatant of CHO-D46 cells, bands around 17 kDa were rarely detected (see for example Figure 16), despite precipitating similar amounts of media. Given that the signal of the ~43 kDa band in Figure 19 is so much more intense than the signal of the smaller bands, it is not surprising that no smaller bands can be seen in Figure 16 or similar blots, as the signal of the high molecular weight bands is already much weaker in that case. The exact reasons for this however remain unknown.

It was discovered later, that the (largely) insoluble fraction resulting from precipitation with EtOH contained significant amounts of activin. Loading both the (urea) soluble and insoluble fractions together on gel (after the addition of *SDS-PAGE loading buffer*) repeatedly resulted in a much more intense signal, similar to that shown in Figure 19.

Even more difficult is the interpretation of the results from gel electrophoresis under non-reducing conditions (see Figure 19 right). It seems that while some protein still exists in the unprocessed but monomeric form (around 43 kDa), a large part forms aggregates of several hundred kDa (compare to activin beta E - GFP below).

Western blotting with anti-GFP antibody of precipitate from activin beta E - GFP expressing cells (CHO-D57) in general gave similar results (see

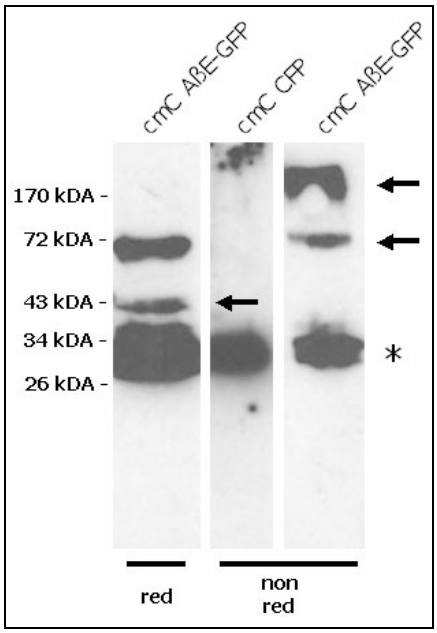


Figure 20, left) as blotting of the HA-tagged variant. A band for the proform (66.2 kDa) was easily detected below 72 kDa under reducing and non-reducing

conditions. While a significant part of the protein seems to form a large complex under non-reducing conditions, preparing the sample in the presence of reducing agents results in a signal a little below 43 kDa which is likely to be caused by the mature monomeric fusion protein (40.1 kDa). The large band below 34 kDa is most likely caused by a contamination of the cell pool with GFP expressing cells. Evidence for this kind of contamination (fluorescent signal in the whole body of the cell as opposed to the secretory pathway only in some cells) was also observed by fluorescence microscopy (not shown).

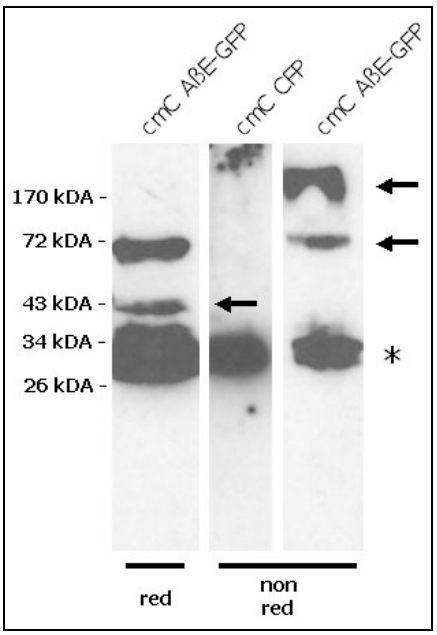


Figure 20 supernatants from CHO cells expressing activin beta E - GFP
media from CHO cells stably expressing activin beta E – GFP (cmC AßE-GFP; 5 mL, 1 T25 flask) as well as from CHO cells stably expressing CFP (cmC CFP) were precipitated and subjected to gel electrophoresis and western blotting under reducing and non-reducing conditions; membranes were probed with antibody against GFP; bands likely to be caused by activin variants are marked by arrows, while an asterisk designates a band probably caused by GFP only (see text)

3.4.3. Pulldown

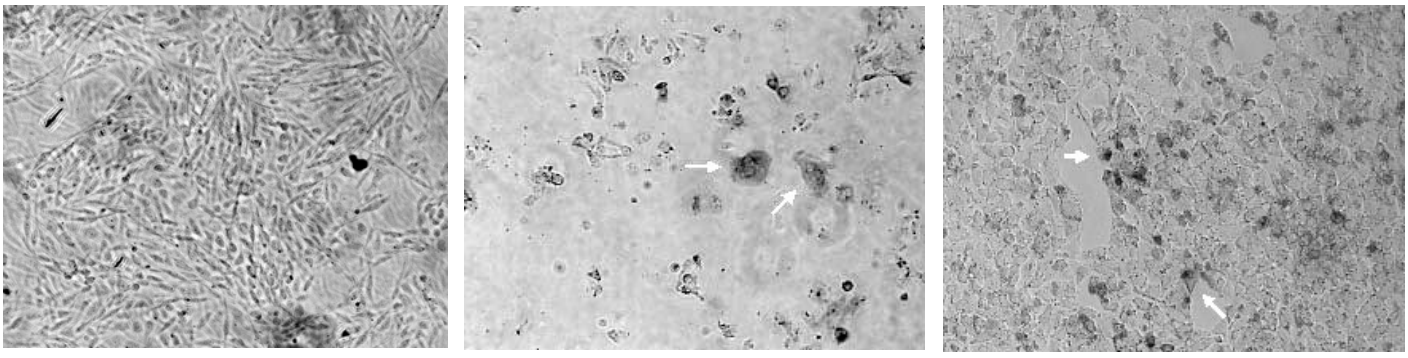
In order to purify activin beta E, and even be able to co-precipitate a possible receptor, preliminary pull-down experiments were undertaken using anti-HA-agarose beads. Early experiments gave promising results under reducing condition (see Figure 19, right). It was possible to precipitate proteins previously identified as proform and mature form of activin beta E – HA (marked by arrows, see above). The strong band identified by an asterisk is most likely caused by fragments of the

antibody eluted from the beads by boiling them in SDS-PAGE loading buffer containing 2-mercaptoethanol. The band was also found after incubation of the beads with control supernatants (not shown). Attempts at using less aggressive methods for eluting the bound protein (HA-peptide, acidic pH) showed that optimization of the methods used would be necessary. The same was true for eluting bound protein by boiling the beads in non-reducing SDS-PAGE buffer which resulted in a strong signal spreading over a large area in the range of >100 kDa, again indicating the formation of aggregates.

3.4.4. Immunohistochemistry and fluorescence microscopy

To test the expression of the gene of interest despite initial problems with the analysis of supernatants, we performed immunohistochemistry and fluorescence microscopy on stable cell lines and baculovirus transduced cells.

While the use of antibodies directed against wildtype activin beta E failed in initial experiments (data not shown), it was possible to show that CHO cells stably transfected with the *D46* plasmid and HepG2 cells transfected with the *G12* baculovirus indeed expressed the HA epitope.



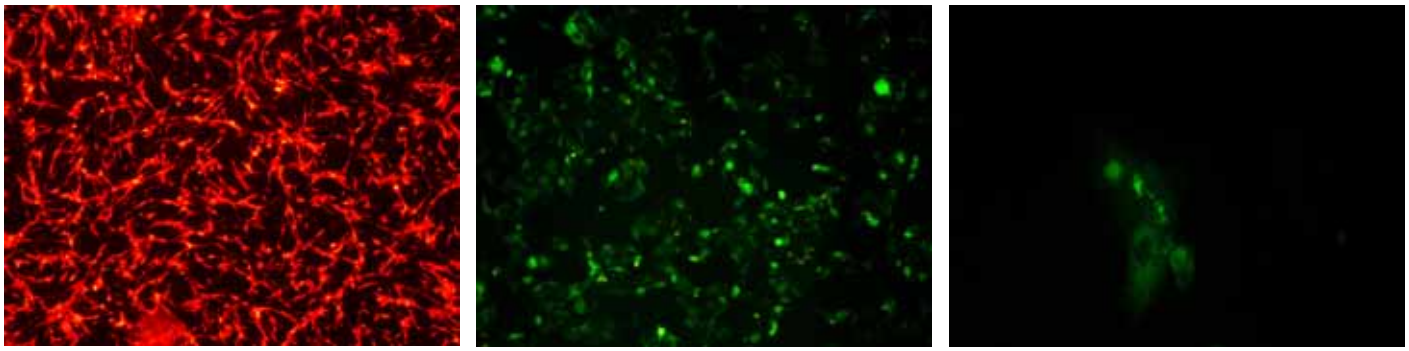
CHO-DHFR$^-$ CHO-D46 HepG2-G12

Figure 21 immunohistochemical analysis of CHO cell lines and baculovirus transduced HepG2 cells

untransfected CHO cells (left), CHO cells expressing activin beta E - HA (middle) and HepG2 cells transduced with baculovirus containing an expression cassette for the same protein (right) were all stained with anti-HA antibody; examples of positives cells are marked by white arrows; contrast increased

In baculovirus transduced cells, transcription of the mRNA containing the gene of interest could also be assessed by fluorescence microscopy, as in most cases, a GFP

containing fusion protein was also encoded on the same mRNA as the gene of interest (see 3.3). In case of the fusion protein between activin beta E and GFP (encoded on *D57*, see 3.1.1), expression of the protein could be visualized directly. See Figure 22.



MGC-G1 MGC-G12 CHO-D57

Figure 22 fluorescence microscopy of virus transduced MGC cells and stable CHO cell lines
MGC cells transduced with G1 virus containing an expression cassette for DsRed, used as control (left), HepG2 cells transduced with G12 virus for expression of activin beta E – HA (+ IRES EGFP-puro) (middle), and CHO cells stably expressing activin beta E – GFP under MTX selection (20nM; right) were examined by fluorescence microscopy**;** contrast increased

3.5. Effects of Activins A and E and their combination on different cell lines

3.5.1. Growth curves

In a growth curve experiment, the effects of stable overexpression of different variants of activin E, in particular the HA-tagged version and the wildtype version, on the proliferation of CHO cells were determined.

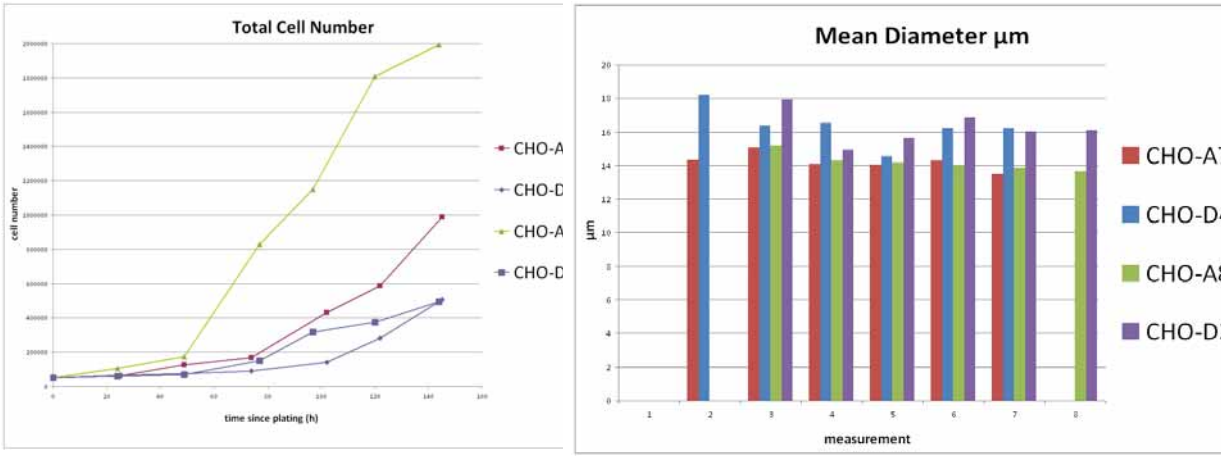


Figure 23 cell proliferation and cell diameter of CHO cell lines
on day one, 50 000 cells per 6-well of the cell lines expressing activin beta E (CHO-D32), activin beta E - HA (CHO-D46) and two mock transfected cell lines (CHO-A8 and CHO-A71) were seeded each in six 6-wells in full medium. CHO-A71 and CHO-D46 were maintained under the selection antibiotic puromycin while CHO-A8 and CHO-D32 were grown in full medium only. Every day, cells from one 6-well each were detached by trypsinization and counted and measured

Figure 23 clearly shows, that CHO cells overexpressing variants of activin E respond with reduced proliferation and increased cells size. The effect on cell proliferation is much more pronounced in the absence of a selection antibiotic, which has a strong anti-proliferative effect on its own (compare for example cell numbers for the two mock cell lines CHO-A8 and CHO-A71 cell lines).

As it has previously been shown, that addition of activin A to the medium inhibits the proliferation of HepG2 cells (Razanajaona, Joguet et al., 2007), we determined if incubating HepG2 and Hep3B cells with conditioned media from the CHO cell lines expressing activin beta E and activin beta E – HA (CHO-D32 and CHO-D46 respectively) and the two corresponding mock cell lines CHO-A8 and CHO-A71 (see Table 11) would influence the growth behavior of the incubated cells.

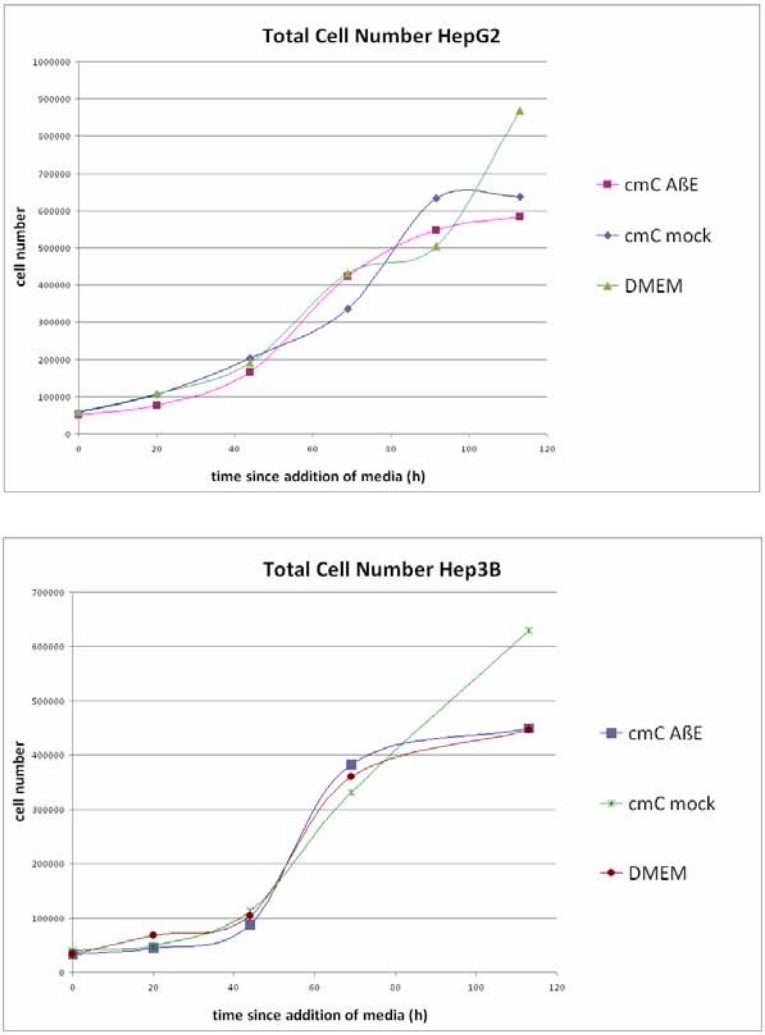


Figure 24 growth curves for HepG2 and Hep3B cells incubated with CHO cell line supernatants
25 000 HepG2 or Hep3B cells were seeded per 12-well in full medium and left to attach for 24 h. Medium was then removed, cells were washed and supernatant of CHO cell lines (10% FBS), diluted 1:3 with fresh growth medium with serum was added to the cells. The following days, every day one well each was harvested and the cells were counted; cmC conditioned medium from CHO cell lines, either expressing activin beta E (AßE) or transfected with a mock plasmid (mock); DMEM, fresh medium with 10% serum

In both cases no clear difference between the effects of control and activin E containing supernatants could be seen. This in accordance with the data from the smad phosphorylation assay (see below) which (in contrast to the effects of activin A) does not show activation of the smad 2 signaling cascade, generally associated with inhibition of cell proliferation (Razanajaona, Joguet et al., 2007).

3.5.2. K562 differentiation assay

It has long been known, that activin A induces erythroid differentiation in the erythroleukemia cell line K562 (Schwall, Nikolics et al., 1988; Shav-Tal & Zipori, 2002).

This can easily be visualized and quantified by staining the cells with 3,3'-dimethoxybenzidine for hemoglobin which accumulates as a consequence of differentiation. For that reason, the assay is frequently used to determine influences on activin A signaling (Lebrun & Vale, 1997; Wang, Huang et al., 2008).

In a first test of the assay outlined in the methods section, we could show that treatment with hemin (50 µM), suggested by Zhang et al. (Zhang, Cho et al., 2007), resulted in a tenfold increase in the number of cells stained positive with benzidine, while treatment with activin A (25 ng/mL) resulted in a threefold increase after five days in culture (see Figure 25). The latter is in accordance with the induction suggested by Wang et al. (Wang, Huang et al., 2008), while for hemin significantly higher numbers have been reported (Nagai, Shimizu et al., 2007).

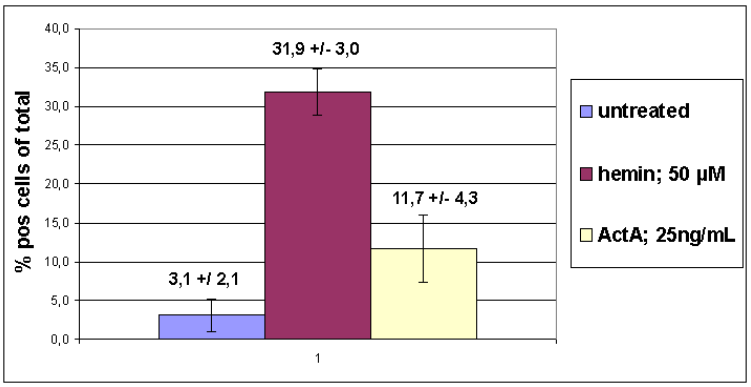


Figure 25 percentage of benzidine positive cells after treatment with hemin or activin A
K562 cells were seeded at a density of 50 000 cells per 24-well into RPMI medium with 10% FBS or RPMI 10% FBS with 50 µM hemin or RPMI 10% FBS with 25 ng/mL activin A; after 5 days, cells were stained for hemoglobin

We then went on to test if supernatants of cells expressing different variants of activin beta E would also cause differentiation of K562 cells, or else influence the effects caused by activin A. To test this, K562 cells were incubated with conditioned media from virus transduced HepG2 cells (at that time, it was not known that in many, but not all cases, supernatant from virus transduced HepG2 cells would only contain biologically inactive activin A – see 3.5.3.1) or supernatant from CHO cell

lines. Since we found that incubating K562 cells with serum free medium resulted in the death of the majority of the cells, serum free HepG2 supernatants were diluted 1:2 with MNP medium with 10% FBS while the supernatant from CHO cells contained 10% FBS from the beginning. A summary of the results is shown in Figure 26.

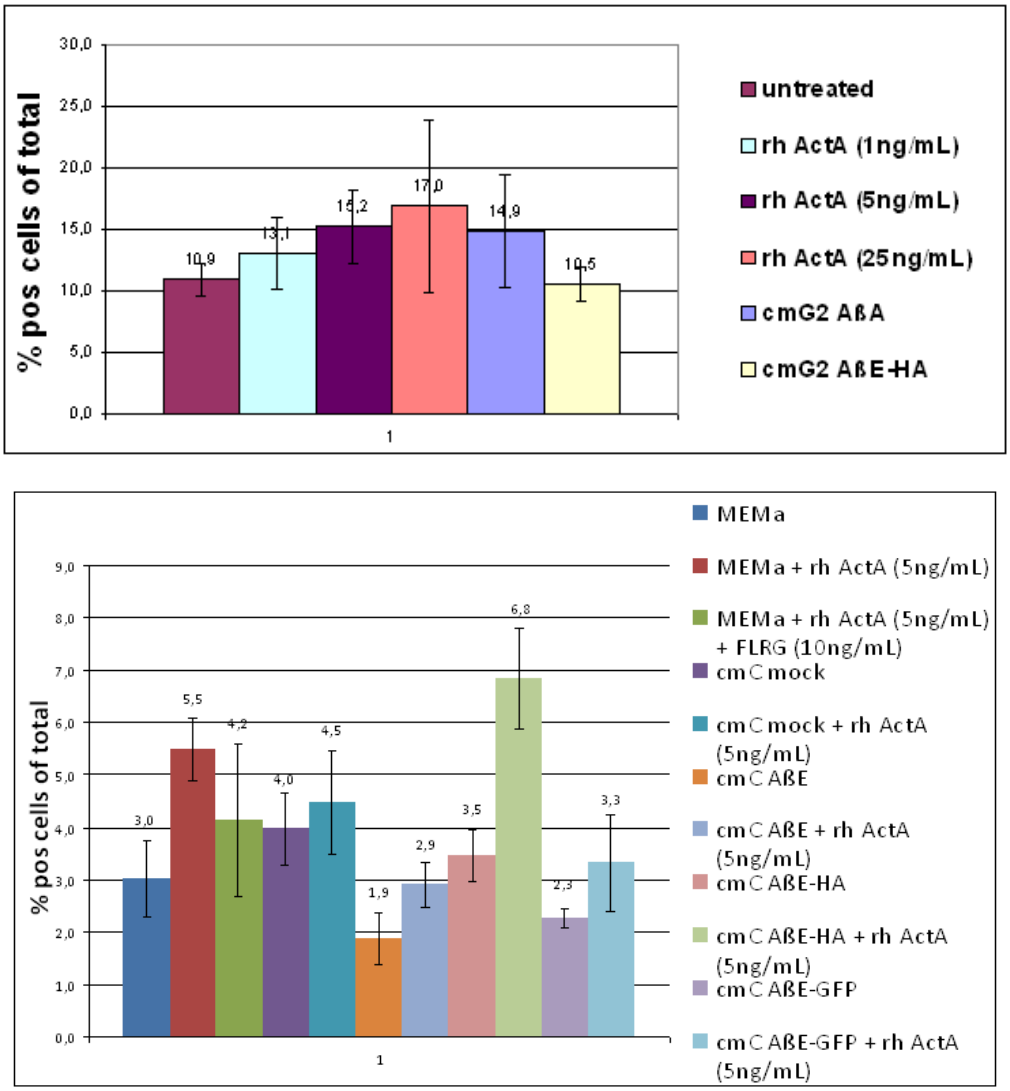


Figure 26 effect of activin E containing supernatants produced in HepG2 cells and CHO cells on the differentiation of K562 cells

top: K562 cells were incubated with conditioned media from virus transduced HepG2 cells either expressing activin beta A (cmG2 AßA) or activin beta E – HA (cmG2 AßE-HA); recombinant activin A (rh ActA) served as a positive control;

bottom: K562 cells were incubated with conditioned media from CHO cell lines either mock transfected (cmC mock), or expressing different variants of activin beta E (cmC AßE; cmC AßE-HA; cmC AßE-GFP); to test for an interplay, recombinant activin A was added to the cell supernatants; fresh CHO medium (MEMa) served as control; FLRG was added as control for the inhibition of activin A; see text for details; staining was performed after 4 days of incubation in both experiments

Although treatment with activin A consistently resulted in an induction of differentiation of K562 cells, the effect caused by small amounts of activin A (for example 5 ng/mL) was rather mild and often almost within the margin of error. Cotreatment with FLRG inhibited the effect of activin A, while activin E variants by themselves had no effect on K562 cells. There was no clear evidence that cotreatment of K562 cells with activin E and activin A would influence the effects of the latter significantly. Only in case of HA-tagged activin E (expressed by the CHO cell line CHO-D46) cotreatment seems to increase the effect of activin A (in comparison to supernatant of the mock transfected CHO cell line CHO-A71, cmC mock). See discussion for details.
Despite the problem with the expression system, activin A produced by baculovirus infected HepG2 cells potently induced differentiation of K562 cells.

3.5.3. Effects of activins on the phosphorylation status of mediators of signaling

3.5.3.1. Smad 2/3

It has previously been shown, that incubation of HepG2 cells with activin A results in phosphorylation of smad 2 which can easily be detected by western blotting of whole cell lysate using commercially available antibodies (Razanajaona, Joguet et al., 2007). We used the same approach to determine if incubating HepG2 cells with activin E would also result in phosphorylation of smad 2, or else influence the activation of the smad signaling cascade by activin A.
We found that even minimal amounts of activin A (down to 0.5 ng/mL in medium; data not shown) resulted in significant phosphorylation of smad 2 after 30 min that could be prevented by adding excess amounts of FLRG to the medium (see Figure 27 and Figure 28 respectively).

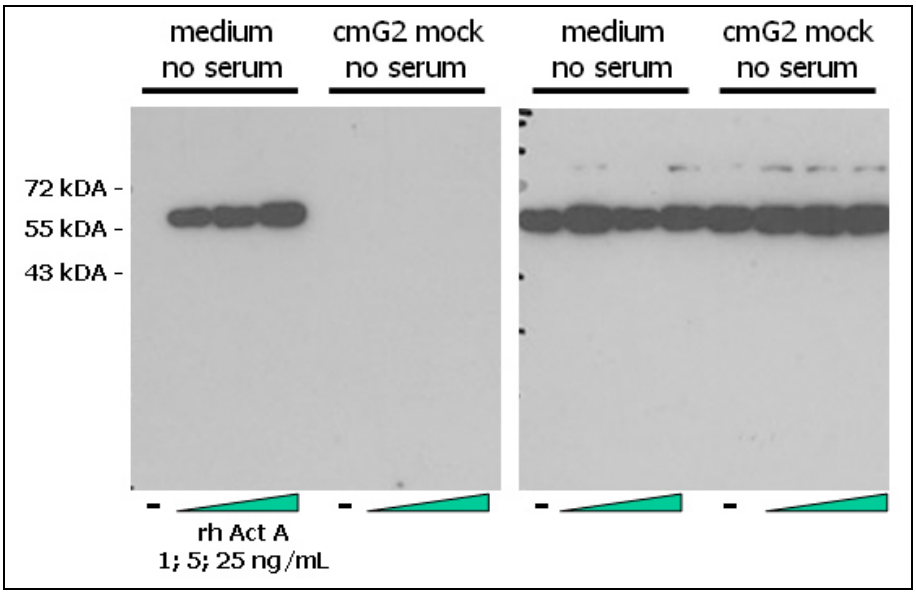


Figure 27 effect of activin A on the phosphorylation status of smad 2 (left) in HepG2 cells and inhibition by HepG2 supernatant; total smad 2/3 (right)
HepG2 cells were treated with increasing concentrations of recombinant activin A (rh Act A), diluted either in serum free medium or conditioned medium from mock virus transduced HepG2 cells (cmG2 mock)

Treating HepG2 cells with supernatants from other HepG2 cells that had been infected with a baculovirus containing an expression cassette for activin A (baculovirus *G9*) unfortunately gave varying results. Supernatants from *G9* infected HepG2 cells induced phosphorylation of smad 2 in some instances while in others they did not (data not shown). HepG2 cells had been chosen as targets for the infection with baculovirus, as they had previously been shown to be particularly suitable for virus mediated expression of a transgene (Boyce & Bucher, 1996). This unexpected observation could be explained by treating HepG2 cells with recombinant activin A and supernatants of mock transfected HepG2 cells at the same time. It could be shown that the supernatant of HepG2 cells was able to block the activity of up to 25 ng/mL activin A (Figure 27). Supernatant from the reporter HepG2 cells themselves did not interfere with activation of signaling as it was removed and the cells were washed directly before the addition of activin A and / or activin containing media (all serum free; see materials and methods).

One possible explanation would be the binding of activin A to alpha 2-macroglobulin produced by HepG2 cells. Alpha 2-macroglobulin has long been known as a binding partner of activin A (Vaughan & Vale, 1993) and has been shown to inhibit binding of TGF-beta to its receptor (Arandjelovic, Freed et al., 2003). While it has been

suggested to aid in clearance of activin A (Niemuller, Randall et al., 1995), other reports have questioned its ability to inhibit biological functions of activin A (Mather, 1996).

Alpha 2-macroglobulin seems to be a particularly likely candidate as it is very abundant in the secretome of HepG2 cells (Christopher Gerner, personal communication).

Another explanation would be the presence of the activin antagonist follistatin which has been found to be expressed by untreated HepG2 cells by some (Rossmanith, Chabicovsky et al., 2002) but not other groups (Bartholin, Maguer-Satta et al., 2002). The other important antagonist of activin A, FLRG, is not expressed significantly by unstimulated HepG2 cells (Razanajaona, Joguet et al., 2007).

The problem could be overcome by changing the expression system from HepG2 cells to MGC cells, a cell line that has recently been shown to be easily transduced with baculovirus (Lackner, Genta et al., 2008).

Treating HepG2 cells with supernatants from MGC cells infected with baculovirus for expression of activin beta A (*G9*), but not treatment with supernatants of MGC cells transduced with a mock virus potently activated the smad 2 pathway (Figure 28).

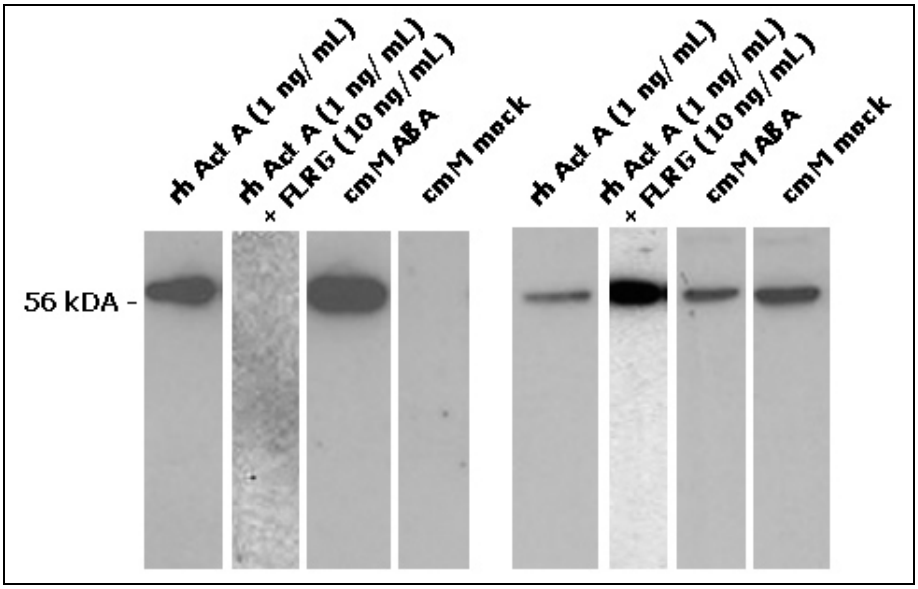


Figure 28 phosphorylation of smad 2 in HepG2 cells by activin A produced in MGC cells (left), total smad 2/3 (right)

HepG2 cells were treated with recombinant activin A (rh ActA), with and without FLRG, or with the supernatants of virus transduced MGC cells expressing either activin beta A (cmM AβA) or a mock construct (cmM MGC)

Once a suitable expression system had been identified, that on the one hand was able to process activins correctly (as shown by production of biologically active activin A) and on the other hand did not interfere significantly with activin A signaling on its own, we went on to determined the effects of artificially expressed activin E on smad 2 protein. It was particularly important to have access to a well tested system for the transient (virus induced) expression of activins, as it was not possible to test if the CHO expression system used would produce cell supernatant containing biologically active activins. As noted before, neither could a stable cell line producing activin A be created, nor could the wildtype activin E be visualized.

In our experiments, we used both supernatants of CHO cells stably expressing different variants of activin beta E, and MGC cells transduced with baculoviruses containing expression cassettes for these proteins. In both cases we found, that treatment of HepG2 cells with activin E (with or without a tag) did not cause detectable smad 2 phosphorylation (see Figure 29 and Figure 30). In addition we tested supernatant from HEK293 cells transiently transfected with the *D32* vector or *D41* vector. The results (not shown) were similar to those for virus transduced and stable cell lines.

As activin E did not cause a detectable phospho-smad 2 signal, we investigated if preincubating HepG2 cells for 30 min with the supernatants of CHO cell lines (Figure 29) or virus transduced MGC cells (Figure 30) before the addition of recombinant activin A would alter the signal. This was of particular interest as it has been suggested that the closely related activin C might act as a competitive inhibitor to activin A (Muenster, Harrison et al., 2005).

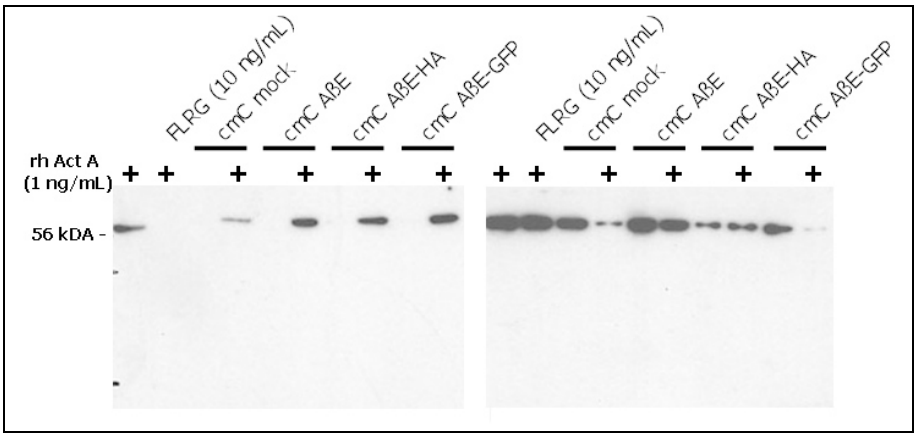


Figure 29 effect of activin E from CHO cells on smad 2 phosphorylation by activin A treatment (left); total smad 2/3 (right)
HepG2 cells were incubated with fresh medium, or conditioned media from CHO cell lines, expressing either a mock construct (cmC mock) or different variants of activin beta E (cmC AßE; cmC AßE-HA; cmC AßE-GFP) for 30 min, before recombinant activin A (rh Act A) was added; addition of FLRG served as a positive control for the inhibition of activin A signaling; an additional 30 min later, cells were harvested and lysed and subjected to gel electrophoresis and western blotting using antibodies against phospho-smad 2 and total smad 2/3

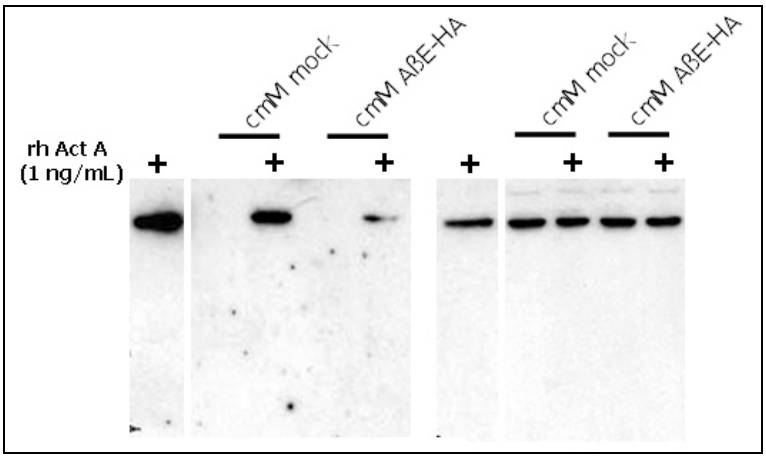


Figure 30 effect of activin E from MGC cells on smad 2 phosphorylation by activin A treatment (left); total smad 2/3 (right)
HepG2 cells were incubated with conditioned media from MGC cells transduced either with a virus containing a mock expression cassette (cmM mock) or an expression cassette for activin beta E – HA (cmM AßE-HA); 30 min later, some of the wells were treated with recombinant activin A (rh Act A); an additional 30 min later, cells were harvested and lysed and subjected to gel electrophoresis and western blotting using antibodies against phospho-smad 2 and total smad 2/3

In Figure 29 it can clearly be seen that neither any of the activin E variants expressed by CHO cells, nor supernatant from mock transfected CHO cells alone has any major effect on the phospho-smad signal caused by as little as 1 ng/mL of activin A. As mentioned before, there is also no evidence for a signal caused by activin E variants alone.

In case of the HA-tagged variant of activin E expressed in MGC cells, a light reduction in signal intensity in comparison to treatment with the supernatant of mock transduced MGC cells can be seen. Taking in consideration however, that supernatant of mock transduced MGC cells alone already causes a decrease in signal intensity, it cannot be ruled out that the additional decrease in case of activin beta E – HA expressing cells is merely caused by differences in the cell number and / or protein synthesis of the cells used for activin expression. Even if that is not the case, the effect of activin E on activin A signaling through smads has to be considered minor at best, judging from the data presented here.

3.5.3.2. Smad 1/5

As it has previously been shown that treatment of responsive cells with the closely related bone morphogenetic proteins (BMPs) or TGF-beta leads to phosphorylation of the smad proteins 1, 5 and 8 (Lee, Ray et al., 2008; Yu, Deng et al., 2008), we went on to investigate if activin E would act in a similar manner. The idea was further supported by the report of experiments in which part of the sequence of activin beta A was replaced with the corresponding regions from BMP-2. This chimerical protein no longer activated the smad 2 signaling pathway but instead caused phosphorylation of smad 1 (Korupolu, Muenster et al., 2008).
Since treatment of the cell line HT1080 (suggested as positive control by the supplier of the antibody raised against phospho-smad 1/5) with purified recombinant BMP-2 (Peprotech EC) did not cause any detectable phospho-smad signal, we went on to use HCT116 cells as reporters (Beck, Jung et al., 2006). Figure 31 shows the phosphorylation status of smad 1 and / or smad 5 in response to treatment with recombinant BMP-2 and supernatant from HEK293 cells transiently transfected with expression vectors for a mock construct protein (EGFP), activin beta E or activin beta A. Before the experiment, the cells had been starved in medium without serum for 1 day. Despite this, a band of similar intensity could be detected in all samples probed with antibody against phospho-smads 1 and 5. As it was not possible to see a difference even between negative and positive control (medium without

serum and BMP-2 respectively), determination of the effects of activin E on the phosphorylation of the smad proteins 1 or 5 has to await optimization of the assay.

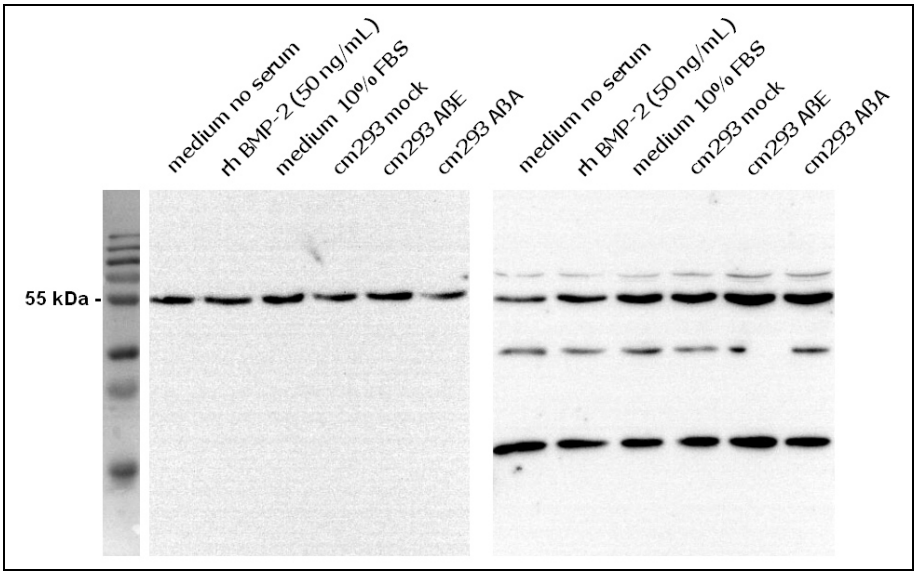


Figure 31 phosphorylation of smad 1/5 (left), total smad 5 (right) from HCT116 cells
HCT116 cells were incubated either with serum free medium, serum free medium with recombinant BMP-2 (rh BMP-2), medium with 10% FBS, or conditioned medium from HEK293 cells transiently transfected with a mock expression vector (cm293 mock), an expression vector for activin beta E (cm293 AßE) or an expression vector for activin beta A (AßA) for 30 min; cells were then harvested and lysed and subjected to gel electrophoresis and western blotting using antibodies against phospho-smad 1/5 and total smad 5

3.5.3.3. MAP kinases and S6 protein

In the course of investigating the effects of activins on the phosphorylation status of smad 2 in HepG2 cells, we also assessed the phosphorylation of ERK 1 and ERK 2 as well as ribosomal protein S6. No difference could be seen between different samples in case of S6 protein (not shown), probably caused by the experimental setup which did not include starving (Sonvilla, Allerstorfer et al., 2008). In case of ERK 1/2 phosphorylation, the results were very inconsistent (not shown), which is likely to have been caused by the different kinetics of ERK and smad phosphorylation (Dupont, McNeilly et al., 2003), and the fact that the experiment had been optimized for the investigation of smad signaling.

3.5.4. Receptor internalization

One approach to identify a possible receptor for activin E was the visualization of receptor-internalization in response to treatment with the ligand. To do so, members of the lab of Michael Grusch had created chimeras between various receptors for members of the TGF-beta family, and fluorescent proteins. A similar approach had previously been described for the receptor of epidermal growth factor (EGF) (Carter & Sorkin, 1998).

Transfection of HEK293 and CHO cells with one or several of the constructs resulted in the expression of fluorescent proteins correctly localized to the membrane in only a tiny fraction of the cells. Most of the cells expressing the constructs reacted by apoptosis a few days after transfection. Similar observations have been made by other groups (Chen, Woodruff et al., 2000).

In preliminary experiments, HEK293 and CHO cells transfected with an expression vector for an EGF-receptor-EGFP chimera failed to react by visible internalization of the membrane protein following treatment with recombinant EGF (20 ng/mL; Peprotech EC) – see Figure 32. While in HEK293 cells, this may be attributed to the reported very low capacity of the endocytotic machinery of these cells (Carter & Sorkin, 1998), the reason for a lack of internalization in CHO cells remains unknown as the process has been shown to work efficiently in this cell line (Szymkiewicz, Kowanetz et al., 2002).

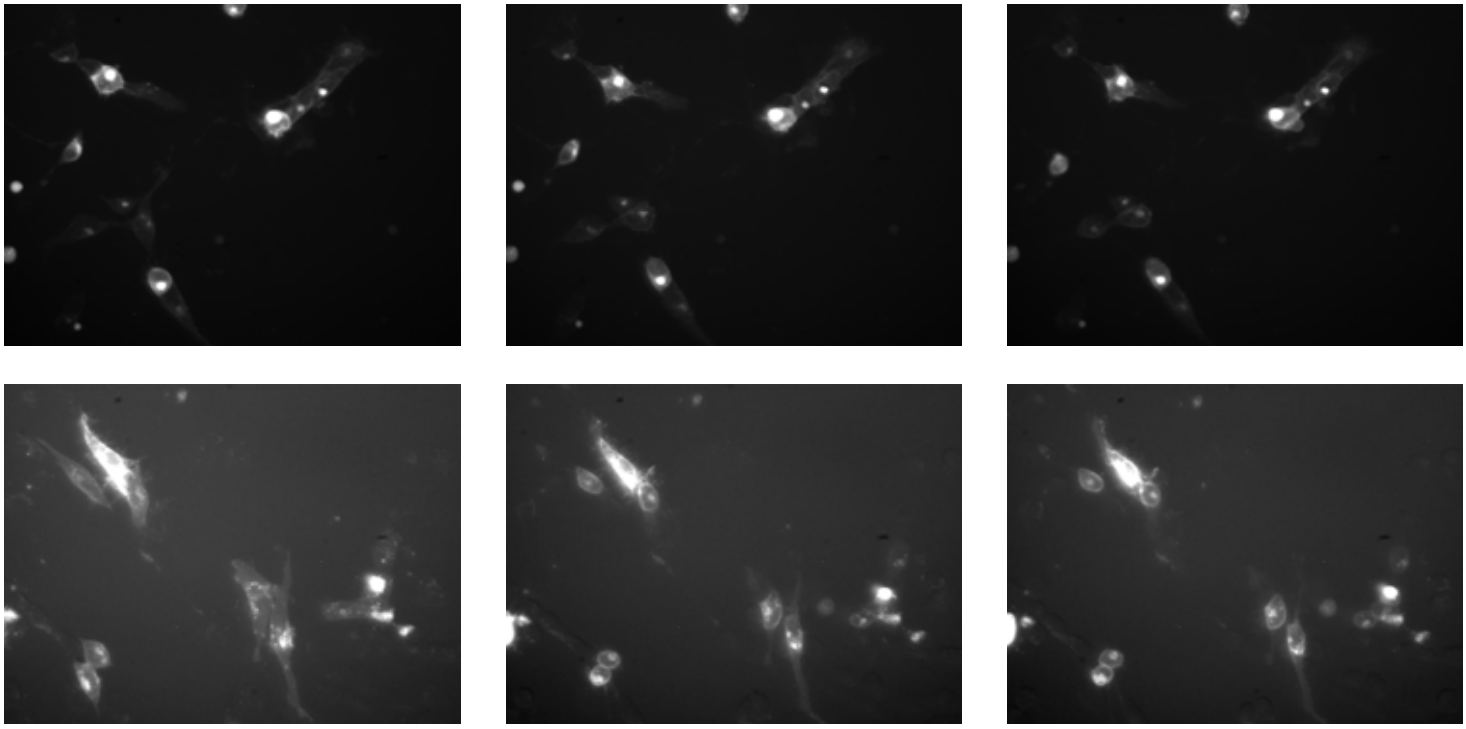


Figure 32 HEK293 (top) and CHO cells (bottom) expressing an EGFP tagged EGF receptor, 1 (left) 45 (middle) and 90 minutes (right) after stimulation with recombinant EGF (20 ng/mL))

Despite the toxicity of the product, it was possible to create cell lines stably expressing BMP receptor 1B - EYFP and activin receptor 2B – ECFP by subcloning or FACS sorting. These may be used in future experiments for the co-precipitation and identification of a bound ligand (Harrison, Gray et al., 2004; Lebrun & Vale, 1997; van der Wijk, Blanchetot et al., 2005).

4. Discussion

4.1. Expression of activin beta E and its tagged variants

The results presented in this thesis suggest that, the CHO expression system and the *BacMam* viral expression system work well for the biosynthesis of activins. Especially the production of bioactive activin A proves that the viral expression system should also be able to produce activin E that has (at least in parts) been correctly processed. Previous reports of the synthesis of bioactive activin A by CHO cells (Arai, Tsuchida et al., 2006; Wada, Maeshima et al., 2004) make it appear likely that our CHO expression system is also capable of producing functional activin E.

The absence of a CHO cell line expressing activin beta A and the unclear results from gel electrophoresis of activin E containing supernatants under non-reducing conditions however made final validation of the suitability of this expression system impossible so far.

In general it was found that the resolubilization of EtOH precipitated proteins is not an easy task. Even in sample buffer containing 8 M urea, a large portion of the precipitate would not go into solution. Even when it did, some of the protein seemed to remain in large aggregates. This was for example seen in case of precipitated DsRed protein which migrated in a band of intense pink above 100 kDa despite the presence of a strong denaturing agent, DTT and 2-mercaptoethanol in the sample. This property of DsRed has been described before for non-reducing gel electrophoresis (Baird, Zacharias et al., 2000). It may well be, that this problem can be overcome by the use of different precipitation techniques and / or optimizations. Using different techniques for the enrichment of activin E in the sample, like classical chromatographical methods used for the purification of activin A (Smith, Yaqoob et al., 1988) or affinity purification used in case of different activin chimeras (Korupolu, Muenster et al., 2008; Muenster, Harrison et al., 2005) would probably avoid this altogether. Early attempts at the latter showed that this approach could in principal be successfully applied to HA-tagged (and GFP-tagged) activin E but also required optimization.

It has to be noted however, that while the CHO expression system could not be validated to 100% so far, data obtained by the use of conditioned media from

transiently transfected and virally transduced cell lines which were shown to be able to express and process activins successfully (HEK293 and MGC respectively) was similar to results from experiments with CHO cell lines.

The ability of the Sf9 expression system to produce correctly processed activin E – although previously shown for activin A (Wuytens, Verschueren et al., 1999) – remains to be tested. An early western blot (not shown) however indicated that the cells were able to produce large amounts of HA tagged protein that also tended to form different aggregates.

We and others (Antenos, Stemler et al., 2007; Arai, Tsuchida et al., 2006; Hashimoto, Ushiro et al., 2006; Mellor, Cranfield et al., 2000; Ushiro, Hashimoto et al., 2006) also identified prominent high molecular weight bands (though with varying intensities in comparison to the mature peptide) corresponding to the proform of the respective protein subunits in the supernatants of different cells ectopically expressing different activins. If the large portion of proform activin beta E, especially in the CHO system is caused by the reported low levels of the (prospective) converting enzyme furin (Ayoubi, Meulemans et al., 1996) remains unknown. Co-expression of furin / SPC1 or SPC 5 or 7 together with activin beta E – HA in CHO cells however did not noticeably shift the balance away from the proform towards the mature peptide (not shown).

4.2. Biological properties of activin E

A basic evaluation of the growth characteristics of CHO cell lines stably expressing activin beta E or tagged variants pointed towards an anti-proliferative effect of the protein in the expressing cell. This is in general accordance with observations made previously (Chabicovsky, Herkner et al., 2003; Vejda, Erlach et al., 2003). It has to be noted however, that this might at least in part be due to the stress upon the ER of cells highly overexpressing this secreted protein. A comparison with cell lines overexpressing other secreted proteins or even a biologically inactive variant of activin A (Fischer, Park et al., 2003; Wuytens, Verschueren et al., 1999) could help answering this question.

Treatment of the hepatoma cell lines HepG2 and Hep3B with activin E containing media however showed no strong inhibition of the proliferation of these cells as it

had been reported for activin A (Chen, Woodruff et al., 2000; Razanajaona, Joguet et al., 2007). This fits well with the observations made in K562 differentiation- and smad phosphorylation assays.

As already mentioned in the results section, the determination of the effects of activins on K562 cells was rather difficult. This was mainly due to the high variability of the results. The fact that conditioned media rather than purified activin E had to be used for these long term experiments was definitely one of the causes of this. On the one hand did the supernatants quite certainly contain rather different concentrations of the protein of interest as well as additional factors secreted and / or released by the producer cell lines. On the other hand did activin beta E producing- and mock transfected cell lines have a different consumption of their growth medium and growth factors from the serum due to highly different proliferation rates. This could already be told from the fairly different color of the supernatants from different cell lines. While it would be possible to overcome this particular problem by dialysis of cell supernatant against fresh medium, the presence of different amounts of other proteins that might have an effect on K562 cells (especially given the long incubation time), could only be avoided by purification of activin E.

Nonetheless, there is no evidence that secreted activin E alone has any major effect on the differentiation of K562 cells toward the erythroid lineage.

Similar results were found for the related activin C which did not cause activation of activin A responsive elements in reporter gene assays (Mellor, Ball et al., 2003). As it has been suggested that this protein might act as competitor of activin A for the binding to the receptor ActRII (Muenster, Harrison et al., 2005), we performed the co-incubation experiments described in the results section.

Results from cotreatment of the erythroleukemia cells with activin A and activin E however were less clear than results from treatment of the cells with one or the other protein alone. This was especially the case, as incubation of K562 cells with activin A in the supernatant of mock transfected cells had only a minor effect, while incubation with the supernatant activin beta E – HA expressing cells and activin A caused a major induction of differentiation. One could speculate that this effect is caused by the depletion through activin E of some inhibitor of activin A produced by CHO cells (see below). The concerns mentioned above however, apply particularly

well to the mock transfected CHO cell line and the CHO cell line producing activin beta E – HA (compare for example light microscopy images of the cells – Figure 13). Although this would in theory be supported by data presented in Figure 29, the large margin of error of the K562 assay and the lack of support for this observation by treatment with supernatant from MGC-G12 cells (transduced with a virus containing the same expression cassette) in the smad assay makes this conclusion rather doubtful. The fact that activin A exerts its function in the differentiation of erythroid cells via signaling through serine / threonine transmembrane receptors and smad proteins (Shav-Tal & Zipori, 2002), and that inhibition of this pathway also inhibits the effects of activin A (Lebrun & Vale, 1997; Wang, Huang et al., 2008), supports the assumption that assaying the pathway further upstream, namely at the level of receptor smad phosphorylation, should yield the same information as observing the degree of differentiation of K562 cells.

While we could show that both recombinant activin A and the supernatant from cells expressing activin beta A induce phosphorylation of smad 2 protein in HepG2 cells, supernatants of activin beta E (tagged and untagged) producing cells had no such effect. Since this result was repeatedly shown with conditioned media from several expression systems (baculovirus transduced HepG2 and MGC cells, stable CHO cell lines and transiently transfected HEK293 cells) which were all in principle able to produce bioactive activin A, it can be considered quite reliable. This is especially the case due to the high sensitivity of the system (0.5 ng/mL of recombinant activin A resulted in a clear phospho-smad signal) and good reproducibility of the results. It cannot be ruled out however, that activin E might need other receptors or additional coreceptors no present in either K562 or HepG2 cells for smad activation.
In cotreatment experiments, there was no strong difference in the phosphorylation of smad 2 protein after treatment with activin A diluted in supernatants from mock transfected or activin beta E expressing cell lines. While the assay would certainly not be able to detect very subtle effects of activin E on activin A signaling, it can be expected that the affinity of activin E for (one of) the receptors responsible for binding and signaling of activin A is rather low, if significant at all. Since the concentration of activin A in the medium (1 ng/mL) was close to the minimum

required for detectable phosphorylation, one would expect that addition of significant amounts of a competitive inhibitors would abolish the signal altogether.
Of course this can only be a raw estimation based on the results presented here. Final testing of the affinity of activin E for receptors of activin A (for example by measuring the displacement of radio-labeled activin A (Muenster, Harrison et al., 2005) has to await successful purification and quantification of activin E. Despite this, neither assaying the phosphorylation of smad 2 proteins, nor determining the differentiation of K562 cells gave any indication for a direct inhibition of activin A signaling by activin E.
Induction of phosphorylation of smad 1 and / or smad 5 by activin E could not be thoroughly tested. Although these smads have previously been associated with the BMP branch of the TGF-beta family, it has been shown that even TGF-beta itself can activate this subset of signal mediators depending on the cell type (Schmierer & Hill, 2007). A repetition of the experiment however should help answer this question.

4.3. Identification of a receptor binding Activin E

The most straight forward way to answer which – if any – of the known TGF-beta family receptors binds activin E of course would be immunoprecipitation of ligand receptor complexes. The stability of which could be significantly enhanced by covalent crosslinking (Lebrun & Vale, 1997). Two approaches are generally possible. CFP / YFP tagged receptors could be precipitated following incubation with activin E and then be analyzed for the coprecipitation of the ligand by western blotting (Lebrun & Vale, 1997; Wuytens, Verschueren et al., 1999). Alternatively, lysate of cells that have been treated with activin E could be incubated with antibodies / beads directed at the ligand or its tag. Interaction partners could either be detected using a candidate approach (for each experiment, cells would be transfected with one of the TGF-beta family receptors fused to tag) or by mass spectrometry (Cheeseman & Desai, 2005; Steen & Mann, 2004). The advantage of precipitating the ligand would be that all purified protein has indeed bound to an interaction partner on the cell as unbound activin E would be washed away before crosslinking and precipitation. This would allow precipitating protein from a very large number of cells making detection of a signal more likely.

Such an experiment is currently hindered however by a lack of tools to be used as controls. The main problem is that by the time the experiments for this thesis were finished, no HA-tagged activin A was available. Since it is currently only possible to detect and / or pulldown tagged activin E, it would first be essential to show that the presence of this tag does not automatically render the protein inactive or alter its receptor specificity. Secondly, it would be important to show that HA-tagged activin can be used either to co-precipitate its receptor or be detected following pulldown of the receptor.

In a preliminary experiment, K562 cells were incubated with activin E – HA, washed, and treated with the membrane impermeable crosslinker DTSSP (DiMartino & Kew, 1999). This was followed by pulldown using anti-HA-agarose beads. In a western blot using rat-anti-HA antibody, no clear difference could be seen between the band pattern (most likely caused by eluted antibody from the beads) from negative control (pulldown from K562 lysate that had not been incubated with HA-tagged activin E) and from activin E - HA treated K562 cells (not shown). As no positive control could be used however, this negative result can be seen as a little hint at best.

Another option would be the visualization of binding (and internalization) of activin E to whole cells. While this approach would not allow for the identification of a binding partner, it is probably much simpler to perform than a pulldown. In a similar experiment it has been shown that bound, labeled activin A can be visualized by histological approaches on whole cells (Jullien & Gurdon, 2005). If this could be shown in a similar manner for activin E, it would be an important step forward. Again however this would require either tagged activin A (HA or EGFP) as a positive control, or "pure" activin E that could be labeled by other means.

As it has been shown, that activin A causes internalization of its receptor ActRIIB (Zhou, Scolavino et al., 2004), it should also be possible to visualize this process using tagged versions of the receptor (Jullien & Gurdon, 2005). By one-by-one testing of possible receptors for activin E for internalization following incubation with the latter, it might be possible to identify the binding partner of this protein. The advantage of this approach would be that it could also be performed using conditioned media from cells expressing untagged activin E and (for a positive control) untagged activin A. However, it has already been mentioned in the results

section that early attempts at this assay failed and that a significant degree of optimization might be necessary.

4.4. Other important effects of activin beta E expression

One important question that could not be addressed at all during the course of this diploma thesis was the formation of activin heterodimers containing the beta E subunit. Using a 2D gel electrophoresis approach, the formation of activin AE and activin CE has previously been shown (Vejda, Cranfield et al., 2002). It has been also suggested that heterodimers between activin beta A and activin beta C have distinctly different properties than homodimers of activin beta A. This could be one way of regulating activin A activity (Mellor, Ball et al., 2003). By transducing cells with the two baculoviruses expressing activin beta A and activin beta E – HA described in the results section, followed by pulldown using anti-HA-agarose beads, it should be possible to test activin AE. Assuming that heterodimers can be purified this way, in a next step, their biological properties in comparison to those of activin A could be determined. Again the main prerequisite for this would be the availability of biologically active activin beta A – HA which would serve as a proof that the purification procedure does not render the protein inactive. As an alternative, classical biochemical methods could be used to purify activin heterodimers containing the beta E subunit (Pangas & Woodruff, 2002). This however could only be done in cooperation with a group routinely employing these techniques.

Finally it would be important to know if, and (roughly) with what affinity activin E (or activin beta E containing activins) binds to known antagonists of activin A.

While Hashimoto et al. (Hashimoto, Tsuchida et al., 2002) showed an interaction between activin E and follistatin, no evidence for binding of activin E to follistatin-like 3 / FLRG has been published so far. We and others show (Razanajaona, Joguet et al., 2007) that FLRG inhibits smad phosphorylation after treatment with activin A. A simple competition experiment with smad phosphorylation as readout should easily tell if activin E relieves its more famous relative from repression by its antagonists via binding to the latter and by this inactivating them. Such a mechanism would be most relevant in tissues like the liver,

where activin beta E, beta A and its antagonists are co-expressed and where the expression level of activin beta E by far exceeds that of activin beta A.

5. List of abbreviations

ActR – activin receptor

ACVR – activin receptor

ALK – activin receptor–like kinase

APS – ammonium persulfate

BMP – bone morphogenetic protein

BSA – bovine serum albumin

CFP – cyan fluorescent protein

CIAP – calf intestinal alkaline phosphatase

cm293 – conditioned medium from HEK293 cells

cmC – conditioned medium from CHO cells

cmG2 – conditioned medium from HepG2 cells

cmM – conditioned medium from MGC cells

DHFR – dihydrofolate reductase

DMSO – dimethyl sulfoxide

DTSSP – 3,3´-dithiobis[sulfosuccinimidylpropionate]

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

EtOH – ethanol

FBS – fetal bovine serum

FLRG – follistatin–related gene

GFP / EGFP – green fluorescent protein / enhanced green fluorescent protein

GOI – gene of interest

HA – hemagglutinin

HT – hypoxanthine

IHC – immunohistochemistry

IPTG – sopropyl β–D–1–thiogalactopyranoside

IRES – internal ribosome entry site

LB – lysogeny broth (aka luria broth)[2]

MAP kinase – mitogen–activated protein (MAP) kinase

MeOH – methanol

MOI – multiplicity of infection

MTX – methotrexate

o/n – overnight

P/S – penicillin, streptomycin

[2] Bertani, G. (2004). Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. *J Bacteriol*, **186,** 595-600.

PBS – phosphate buffered saline

PFA – paraformaldehyde

Pfu – plaque forming units

PNK – T4 polynucleotide kinase

PVDF – polyvinylidene fluoride

rh Act A – recombinant human activin A

RT – room temperature

SDS – sodium dodecyl sulfate

SDS–PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOB – super optimal broth

SOC – SOB with "B" changed to "C" for catabolite repression because of added glucose

STET – sodium chloride Tris EDTA Triton X–100 buffer solution

T/E – trypsin/EDTA

TBE – Tris/borate/EDTA

TBS – Tris buffered saline

TCA – trichloroacetic acid

TE – Tris EDTA buffer solution

TEMED – N,N,N',N'–tetramethylethylenediamine

TGF–beta – transforming growth factor beta

Tris – tris(hydroxymethyl)aminomethane

X–gal – 5–bromo–4–chloro–3–indolyl beta–D–galactopyranoside

YFP – yellow fluorescent protein

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Education

2003 – 2008	Student of Molecular Biology, University of Vienna
1994 – 2002	Highschool: Lise-Meitner Realgymnasium, Vienna
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Research

September 2007 –	Michael Grusch Group Institute of Cancer Research Medical University Vienna **Diploma Thesis:** "The Role of Activin Beta E in Receptor Mediated Signaling"
April 2007 – May 2007	Frank Hilberg Group Boehringer Ingelheim Austria Inhibition of Angiogenesis in Cancer – Small Molecule Inhibitors
January 2007 – March 2007	Jan-Michael Peters Group IMP Vienna MitoCheck Project
August 2006 – September 2006	Michael Grusch Group Institute of Cancer Research Medical University Vienna

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July 2006 – August 2006	Franz Koller Group Department of Biochemistry University of Vienna Protein-engineering of Hydroperoxidases
April 2006 – May 2006	Erwin Ivessa Group Department of Medical Biochemistry Medical University Vienna Synthesis, folding, transport and degradation of proteins in the early secretory pathway

Publications

Deli A, Kreidl E, Santifaller S, Trotter B, Seir K, Berger W, Schulte-Hermann R, Rodgarkia-Dara C, Grusch M.
Activins and activin antagonists in hepatocellular carcinoma.
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