Identification and characterisation of HDGF splice variants and their differential secretion via exosomal microvesicles

Dissertation
zur Erlangung des Doktorgrades der Naturwissenschaften
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Juni/Juli 2016
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Datum des Kolloquiums: 15.08.2016
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I. Summary

Hepatoma-derived growth factor (HDGF) is a ubiquitously expressed protein and involved in a variety of cellular processes. Since HDGF exhibits growth stimulating activity, research focused mainly on its role in cancer and tumour biology.

Interestingly, screening the NCBI database for HDGF entries led to the discovery of two further alternative human HDGF isoforms, termed HDGF-B and HDGF-C in the present work. Even though these entries have been present in the NCBI database for quite a while, the respective HDGF isoforms have been neglected in HDGF research by now. Therefore, main aim of this thesis was the verification and initial characterisation of those two novel HDGF isoforms.

HDGF-B and HDGF-C arise from the usage of an alternative first in-frame exon and the expressed proteins exhibit changes in their N-terminal part. The remaining C-terminal part is identical to that of the basic HDGF form, now termed HDGF-A.

Since most of the known interactions and functions of HDGF-A have been assigned to its N-terminal part, changes in this part of the protein might have a huge impact. Indeed, the results of this thesis indicated that the novel isoforms carry out quite distinct functions. This became particularly obvious regarding the analysis of co-precipitated proteins of each isoform. Only HDGF-A co-precipitated the RNA binding proteins nucleolin and YB-1, whereas HDGF-B and HDGF-C lost these interaction partners. Instead, these isoforms were able to co-precipitate tubulin and the motorprotein dynein and conversely these interaction partners were not detectable in the co-precipitate of HDGF-A.

A further alteration was discovered in the secretion behaviour of these isoforms. HDGF-A is considered as a protein which leaves the cell via an alternative secretion pathway. Recently, some major proteomic studies detected its presence in exosomes, small secreted vesicles. This discovery was supported by the results of this thesis. Moreover, it was found that HDGF-A is located within the lumen of those extracellular vesicles. Interestingly, HDGF-A was additionally detected as free protein in the surrounding media, suggesting that it uses an additional alternative pathway to leave the cell. By contrast to this the novel isoforms HDGF-B and HDGF-C were detected at the surface of extracellular vesicles without any evidence that these proteins are additionally present as free protein.

A remarkable finding of this thesis was the discovery that truncation of the outermost 15 N-terminal HDGF-A is sufficient to induce the same secretion and interaction behaviour as observed for HDGF-B and HDGF-C. The results highlighted the importance of the outermost N-terminal peptide in HDGF-A. A previous study revealed that N-terminal truncation might be controlled by dephosphorylation of serine residue 165 in HDGF-A.
Assumed that such a truncation mechanism exists also under physiological conditions the cell is able to control the mode of secretion as well as interaction behaviour not only by alternative splicing but also by posttranslational modifications. This work provides insight into new aspects of HDGF, which help to understand its biological function and open up new perspectives for future studies.
II. Zusammenfassung


Diese beiden Isoformen entstehen durch die Verwendung eines alternativen ersten Exons, welches sich im gleichen Leseraster zu den nachfolgenden kodierenden Exons befindet. Die beiden Isoformen weisen somit Veränderungen im N-terminalen Bereich des Proteins auf, während der übrige Teil identisch zu dem C-terminalen Teil der ursprünglich beschriebenen HDGF Isoform ist, die nun als HDGF-A bezeichnet wird.

Da die meisten der bekannten Interaktionen und Funktionen von HDGF-A dem N-terminalen Bereich zugeschrieben werden, haben Veränderungen in diesem Teil des Proteins möglicherweise große Konsequenzen.


Auch das Sekretionsverhalten der neuen HDGF Isoformen zeigte sich verändert. HDGF-A wird generell als sezerniertes Protein angesehen, welches über alternative Sekretionswege die Zelle verlässt. Kürzlich konnten Massenspektrometriestudien zeigen, dass HDGF sich in Exosomen, kleine sezernierte Vesikel, befindet. Diese Arbeit unterstützte die Entdeckung dieser Studien und konnte darüber hinaus zeigen, dass HDGF-A sich innerhalb dieser Vesikel befindet. Interessanterweise lag HDGF-A auch als freies Protein im Kulturüberstand vor, was darauf hindeutet, dass ein weiterer Sekretionsweg benutzt wird. Einen starken Kontrast dazu bildeten die neuen Isoformen HDGF-B und HDGF-C. Sie befanden sich auf der Oberfläche von extrazellulären Vesikeln und lagen nicht zusätzlich als freie Proteine im Kulturüberstand vor.

Diese Arbeit gibt Einblick in neue Aspekte über HDGF, die dabei helfen seine biologische Rolle zu verstehen und neue Perspektiven für künftige Studien eröffnen.
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V. Abbreviations

aa  amino acid
ABC  adenosine binding cassette
ATP  adenosine triphosphate
bcl-2  B-cell lymphoma 2
BFA  Brefeldin a
BiFC  Bimolecular fluorescence complementation
bp  base pairs
BrdU  bromdesoxyuridin
BSA  bovine serum albumin
cDNA  complementary deoxyribonucleic acid
CL  cleared cell lysate
DAPI  4,6-Diamidino-2-phenylindol
DLS  dynamic light scattering
DMEM  dulbecco's modified serum
DMA  dimethylamiloride
DNA  deoxyribonucleic acid
DTT  dithiothreitol
ECL  enhanced chemiluminescence
EDTA  ethylenediaminetetraacetic acid
EGFP  enhanced green fluorescent protein
EMT  epithel-mesenchymal transition
ER  endoplasmatic reticulum
ERK  extracellular-signal Regulated Kinase
ESCRT  endosomal sorting complex required for transport
EST  expressed sequence tags
EtOH  ethanol
Ex  exosome-containing fraction
EYFP  enhanced yellow fluorescent protein
FCS  fetal calf serum
GCS  glioblastoma cancer stem like cells
hath  homologous to the amino terminus of HDGF
HDGF  hepatoma-derived growth factor
HMGB1  high-mobility group protein-1
HRP  HDGF-related protein
Hsc  heat shock cognate
Hsp  heat shock protein
IF  immunofluorescence
IL  Interleukin
ILV  intraluminal vesicle
kDa  kilo Dalton
LDH  lactate dehydrogenase
LEDGF  lens epithelium-derived growth factor
LPS  lipopolysaccharide
MALDI-TOF MS  Matrix-assisted laser desorption/ionisation - time of flight mass spectrometry
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Mon</td>
<td>Monensin</td>
</tr>
<tr>
<td>MVB</td>
<td>multivesicular body</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mRNP</td>
<td>messenger ribonucleoprotein</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localisation signal</td>
</tr>
<tr>
<td>NMD</td>
<td>nonsense-mediated mRNA decay</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>POD</td>
<td>peroxidase</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cells</td>
</tr>
<tr>
<td>SN</td>
<td>supernatant</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin like modifier</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>YB-1</td>
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1. Introduction

1.1 Hepatoma-derived growth factor

1.1.1 HDGF: Structure and function

The Hepatoma-derived growth factor (HDGF) was originally purified as a secreted factor from the medium of human hepatoma-derived cell line HuH-7 and described as a 25 kDa acidic heparin-binding protein with growth-stimulating activity [1]. Meanwhile it is known, that this protein is expressed in a wide variety of tissues and cell lines [1-3]. HDGF appears to be a multifunctional protein that is involved in numerous cellular processes [4-6]. This is mirrored by the huge number of diverse interactions partners, which were identified in co-precipitates of HDGF. The identified proteins participate in different cellular events such as ribosome biogenesis or mRNA metabolism [5, 7].

HDGF is the first identified member of the HDGF-related protein (HRP) family, a protein family which to date contains five additional members: HRP-1, HRP-2 [8], HRP-3 [9], HRP-4 [10] and lens epithelium-derived growth factor (LEDGF) [11]. As shown in the alignment (figure 1) all members of this family exhibit a high homology in the N-terminal region (amino acid residues 1-98 in HDGF), termed hath region (homologous to the amino terminus of HDGF), while their C-terminus is variable in length and charge [8, 10].

![Sequence alignment of the N-terminal hath regions of the HRP family.](image-url)

**Figure 1: Sequence alignment of the N-terminal hath regions of the HRP family.** Multiple alignments obtained by clustal omega (1.2.1) [12] online tool with default mode. Accession numbers: HDGF: NP_004485.1; LEDGF: AAH64135; HRP-1: EAW55436; HRP-2: NP_001001520.1; HRP-3: NP_057157.1; HRP-4: CAB40348). Amino acid sequences are given in the single letter code. Asterisks (*) indicate positions which have a single, fully conserved residue, double dots (·) indicate conservation between groups of strongly similar properties, single dots indicate conservation between groups of weakly similar properties. The PHWP motif is highlighted by a black box.
While the N-terminal hath region exhibits a well-defined secondary structure, the C-terminal part of HDGF was characterised as disordered [13]. Disordered proteins lack a folded tertiary structure under physiological conditions. This allows a high intramolecular flexibility which can confer advantages over a strict ordered structure in specific protein functions. Indeed, such protein regions gain more and more attention and have been identified in a growing number of proteins that accomplish important cellular functions such as cell cycle control, transcription regulation or signalling functions [14, 15].

Yet, most known interactions of HDGF are assigned to the well structured hath region such as majority of the identified HDGF-protein interactions [5] as well as its DNA binding ability [16]. The exact function and putative interaction partners of the C-terminal part of HDGF are widely unknown. Interestingly, for the mitogenic activity of HDGF the non-hath region appears to be responsible since overexpression of C-terminal non-hath part alone is sufficient to enhance proliferation [17].

The secondary structure of the N-terminal hath region, depicted in figure 2, consists of five β-strands and two α-helices. The five β-strands form an anti-parallel β-barrel followed by the two C-terminal α-helices. The PWWP domain (amino acid residue 12-68 in HDGF), a structural protein domain, is part of this hath region in HDGF. This domain, first characterised from the WHSC1 gene [18, 19], was identified in a wide range of mostly chromatin associated proteins within the eukaryotes [20]. It has a role in protein-protein interactions as well as in different DNA related processes such as transcriptional regulation [21], DNA repair [22] or DNA methylation [23]. The around 70 amino acid (aa) residue long domain was designated PWWP because of its central core motif ‘proline-tryptophan-tryptophan-proline’ [18, 19]. However, the name is slightly deceptive since only the fourth position of this motif (proline) is conserved among all family members [20]. As shown in the alignment, this core motif PWWP is changed to ‘proline-histidine-tryptophan-proline’ (PHWP) motif in HDGF, LEDGF, HRP-2 & HRP-3 and to ‘alanine-histidine-tryptophan-proline’ (AHWP) motif in HRP-1 and HRP-4.

The surface of the hath region exhibits large positively charged patches due to the presence of a series of positively charged amino acid residues. This positively charged surface is responsible for heparin binding [13] as well as for DNA binding [16]. Heparin is a highly negatively charged linear polymer consisting of repeating units of 1→4-linked uronic acid and glucosamine residues [24]. Yang et al. [25] showed that the PWWP domain of HDGF mediates the specific binding to a minimum 37 bp long DNA fragment within the promoter of the SMYD1 gene and thereby functions as a transcription repressor.

Interestingly, the PWWP domain is also responsible for the formation of HDGF dimers via an unusual domain swap mechanism that involves the exchange of the first two N-
terminal β-sheets. Resulting homodimers have higher affinity to heparin [26] but the exact biological role of those dimers is unknown.

Furthermore, HDGF contains two nuclear localisation sequences (NLS) [9, 27]. NLS 1 is located in the hath region (amino acid residues 75-80), while the second bipartite NLS motif is present in the C-terminal non-hath region (amino acid residues 155-170) (figure 2). Studies showed that the presence of the bipartite NLS 2 signal in the C-terminal part is essential for nuclear localisation of HDGF and simultaneously, nuclear entry is required for the mitogenic effect of intracellular HDGF [17, 28].

Despite the fact that HDGF was originally identified to be exclusively present in the cytoplasm [1], the subsequent majority of studies revealed that HDGF is predominantly present in the nucleus of cells [2, 17]. Multiple studies found that HDGF can be additionally detected in the cytoplasm [3, 17, 29-32], indicating that HDGF is able to reside in both compartments.

![Figure 2: Schematic illustration of present domains and motifs in HDGF.](image)

As mentioned before HDGF is able to stimulate the proliferation of multiple cell types including fibroblasts [1], endothelial cells [37, 38] and smooth muscle cells [2]. Moreover, it supports cell migration and invasion [39, 40].

It is not completely understood how exactly HDGF exerts its proliferative activity on cells. In this regard, it is assumed that HDGF acts via two independent pathways [41] as depicted in figure 3. The first pathway requires nuclear localisation and depends on the C-terminal non-hath region [17]. The second pathway involves extracellular HDGF, which mediates its activity via a receptor-mediated pathway. In contrast to this first pathway the second pathway depends on the presence of the amino acid residues 81-100 in HDGF.
These amino acid residues constitute the putative binding site in HDGF for a yet unknown receptor which is coupled to downstream signalling pathways [41]. Recently Chen et al. demonstrated that HDGF can bind surface nucleolin [42]. Whether nucleolin is the exclusive surface receptor or also other receptor can be used is still unclear.

Figure 3: Putative signalling pathways of HDGF. HDGF (grey circles) can stimulate proliferation by two independent pathways. First pathway (1) requires nuclear localisation and the C-terminal hath region. The exact mechanism which leads to cell proliferation is obscure. Second pathway (2) involves extracellular HDGF, which binds to an unknown plasma receptor and by that stimulates signalling pathways (MAPK and PI3K). Furthermore, extracellular HDGF can be internalised by macropinocytosis and its internalisation depends on surface heparin (3). The figure was adapted without permission from [4] with some modifications.

In accordance with the assumption that HDGF acts through a receptor mediated signalling pathway, was the discovery that treatment of endothelial cells with exogenous HDGF initiates mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinases (ERK1/2) pathway already after 5 min of exposure [38, 43]. Also for ovarian cancer cells [44] and in osteosarcoma cells [43] stimulation of MAPK/ERK1/2 upon HDGF exposure was shown. Similarly, overexpression of HDGF led to stimulation of the same signalling pathway [45].

Although Everett et al. excluded stimulation of phosphatidylinositol 3-kinase (PI3K)/Akt signalling in endothelial cells upon exogenous HDGF treatment [38], other studies were able to demonstrate this in osteosarcoma cells [43] as well as in fibroblast cell line.
NIH/3T3 [46], revealing that HDGF can induce more than one intracellular signalling pathway. Interestingly, the neutralisation of surface nucleolin by antibodies led to the decrease of HDGF induced PI3K/Akt signalling [47]. HDGF is a phosphoprotein and, as indicated in figure 2, several different serine residues were experimentally identified as phosphorylation sites in HDGF [36, 48]. However, at the current stage not much is known about functional consequences of this posttranslational modification. Everett et al. could show that the phosphorylation of serine residue 103 enhances the mitogenic activity of HDGF [36]. Phosphorylation or dephosphorylation at serine residue 165 seems to be involved in secretion related process [33]. Apart from phosphorylation sites a SUMOylation site was identified in the N-terminal hath region at lysine residue K80. Strikingly, SUMOylation at this position led to the loss of chromatin association [49].

### 1.1.2 Biological role of HDGF

The biological role of HDGF appears to be linked to embryonic development and regeneration processes. In this context it was shown that HDGF expression is developmentally regulated. In prenatal smooth muscle cells HDGF is highly expressed, while after birth HDGF expression declines [2]. This was also observed for hepatocytes [50]. Furthermore, HDGF is widely distributed within the developing kidney, while it cannot be detected in the adult kidney [37]. Other studies implied that HDGF plays a role in lung [51], liver [50], brain [3], gut [7] and heart [27] development. By contrast, Gallitzendörfer et al. [52] generated HDGF deficient mice, which did not show apparent differences to the wild type mice, indicating that HDGF is dispensable in mice for the development and growth.

Besides, HDGF might be also involved in processes of organ repair and remodelling after injury. In smooth muscle cells HDGF is highly expressed after vascular injury and seems to play a role in their subsequent proliferation [39]. Furthermore, Enomoto et al. suggested that HDGF is involved in liver regeneration as autocrine factor [53]. Similarly it might participate in intestine regeneration [54]. In addition, HDGF is able to promote angiogenesis [2, 38, 55-58].

Like for other growth factors misregulation of HDGF has a significant impact on tumourigenesis and cancer progression. An increasing number of studies demonstrated elevated HDGF expression in a wide variety of human tumours including hepatocellular cancer [31], non-small cell lung cancer [59], pancreatic cancer [60] or gastric cancer [61]. Overexpression of HDGF correlates with aggressiveness of cancer as well as poor clinical outcome and, therefore, HDGF can be considered as a prognostic tool for some cancer types, e.g. non-small cell lung cancer [62], pancreatic cancer [60] or
glioblastomas [63]. In accordance, suppressing HDGF by small interfering RNA (siRNA) lead to slower cell growth [40, 64] and decreased invasiveness [64], as well as reduced metastasis formation [65] which makes HDGF a putative therapeutic target. Recently it was found that HDGF overexpression as well as exogenous HDGF supply induces epithel-mesenchymal transition (EMT) [66]. EMT is a transition process by which epithelia cells lose their typical characteristic such as cell-cell adhesion and cell polarity to become mesenchymal cells, which are featured by more migratory and invasive properties [67]. This process is directly linked to cancer invasiveness and metastasis [68] and it seems that HDGF increases migratory and invasiveness of cancer cells by modulating EMT [65, 66, 69].

Related to its pathophysiological role in different cancers is the fact that HDGF may act as a survival factor. Blocking HDGF by antibodies or siRNA induces apoptosis in HepG2 cells as well as in human colorectal carcinoma cells [70-72] and gastric cancer cells [73].

1.1.3 HDGF and secretion

Several proteins are able to function on both sides of the membrane and thus fulfil extra- and intracellular tasks [74]. Such a dual role was described for heat shock proteins [75] as well as for high-mobility group protein B1 (HMGB1) [76], which shares a high degree of similarity with HDGF particularly in the acidic C-terminal region [1]. Since HDGF exerts its function as intracellular and extracellular protein [17, 41], it can be sorted into the same group of proteins with a dual role.

To realize extracellular tasks, release into the extracellular space is required. Indeed, HDGF was originally isolated from the medium of HuH-7 cells [1] and also other studies demonstrated that HDGF can be found in the surrounding medium of a wide variety of cells including neurons [77], endothelial cells [37], skeletal muscle cells [78] or keloid keratinocytes [79]. In this regard, HDGF was also identified blood serum collected from non-small lung cancer patients and moreover, extracellular HDGF levels were elevated compared to the serum from healthy patients [80]. According to Radisky et al. [74] the release via non-classical secretion pathways is typical for such proteins, which fulfil dual tasks inside and outside of cells. In line with this is the fact that HDGF does not contain a hydrophobic secretion signal which mediates the classical secretion pathway.

Some studies discussed HDGF as a protein which is mainly passively released from cells which undergo necrosis, while it remains in the nucleus of apoptotic cells [44, 77]. Such a passive release was also described for HMGB1 [81] and both proteins were classified as potential alarmins, passively released proteins which are involved in signalling cell and tissue damage to the immune system [82, 83].
Interestingly, HDGF release can be controlled, which suggests an underlying active secretion process. Thakar et al. [33] were able to show that one single putative phosphorylation site in HDGF seems to play a regulative role in its secretion. Serine residue 165 is conserved in human and mouse HDGF and independent studies demonstrated experimentally that in human and murine HDGF serine residue 165 is phosphorylated [34, 48, 84-86].

Strikingly, when changing the serine at position 165 to alanine, the resulting HDGF mutant S165A cannot be detected in the cell supernatant after 24 h, although a clear signal is present in case of HDGF wt [33]. In comparison to the wild type, the S165A mutant has a faster electrophoretic mobility due to a loss of the first N-terminal amino acids. Likewise, truncated HDGF forms deleted in first N-terminal 11 or more amino acids cannot be detected after 24 h in the culture medium. This led to the hypothesis that the loss of phosphorylation site S165 promotes N-terminal truncation and in turn prevents secretion. Interestingly, the N-terminal 10 amino acids long peptide is able to act as mediator for secretion, when fusing it to reporter proteins like EGFP or SNAP [33]. However, the question remains how HDGF leaves the cell. Several alternative secretion pathways have been described in literature. Recently, HDGF was identified in exosomes, small secreted vesicles [87-89]. Because of this, this pathway is described in more detail in the next chapter.

1.2 Alternative secretion: HDGF in exosomes

A universal feature of all cells is that they are surrounded by a membrane, which serves as selective barrier between the environment and the inside of the cell. Proteins, which are destined for secretion, have to cross this barrier [90]. Within the eukaryotic cell several pathways are possible. The majority of secretory proteins contain a specific N-terminal signal sequence, which mediates classical secretion via ER-Golgi dependent route [91]. For an increasing number of proteins, alternative secretion pathways have been identified. These proteins lack the classical signal peptide and their secretion is not inhibited by brefeldin A (BFA) [92], a well known inhibitor of the classical secretion route [93].

A growing number of proteins have been identified in exosomes; including HDGF. Complex proteome analyses have identified HDGF in exosomes collected from colon cancer [89, 94] and ovarian cancer cells [88] leading to its entry in the Exocarta database (www.exocarta.org) [95].

Exosomes are 40-100 nm vesicles, that are released in the extracellular space by the majority of cell types including epithelial cells [96], reticulocytes [97], dendritic cells [98] or various tumour cell lines [99]. To date, exosomes are the only known secreted
vesicles which derive from an internal membrane [100]. As illustrated in figure 4, the biogenesis of exosomes involves the maturation of early endosomes to multivesicular bodies. This maturation process is accompanied by inward budding of endosomal membranes and the subsequent creation of intralumina vesicles (ILV) [101, 102]. ‘Degradative’ MVBs can be targeted for degradation by fusing with lysosomes [103, 104]. On the other side the intraluminal vesicles (ILV), along with their cargo, can be released into the extracellular space as result of the fusion of ‘exocytic’ MVBs and plasma membrane [97, 105]. However, a specific mechanism which determines the fate of the MVBs is still unknown [100].

![Figure 4: Generation of exosomes.](image-url) Early endosomes, generated at the plasma membrane can either fuse with the plasma membrane or form MVBs. Inward budding at the limiting membrane of MVBs results in the formation of intraluminal vesicles (ILV). MVBs can have distinct fates, either they evolve into lysosomes or they can fuse with the plasma membrane and release their ILVs into the extracellular space. Those microvesicles are termed exosomes. Vesicles derived by blebbing of the plasma membrane are termed shedding microvesicles. The illustration was adapted from [106] without permission.

Exosomes contain a unique protein and lipid composition. The exact composition varies depending on the cell type but some proteins such as tetraspanins CD63 or CD81 are specifically enriched in exosomes and are useful as marker proteins [107, 108]. Sorting into ILV is regarded as selective process but underlying mechanisms are not well understood [109]. The best characterised mechanism for incorporation of proteins into ILVs involves a multi-enzyme complex termed endosomal sorting complex required for transport (ESCRT). Cargo proteins, which are incorporated by this mechanism, are usually ubiquitinated [110]. This sorting process was mainly described for proteins, which are destined for the degradative MVBs [109, 111] and its involvement in exosome
secretion is still unclear. Moreover, a tetraspanin and lipid-dependent mechanism which is independent from the ESCRT machinery, has been described [112].

The biological role of exosomes is diverse. Early studies of reticulocytes indicated that the secretion of exosomes is an alternative way to discard obsolete membrane proteins such as transferrin receptors [97]. To date it becomes more and more evident, that exosomes participate in cell-cell communication. Recent studies demonstrated that exosomes are not only able to transfer proteins and lipids, but are also able to deliver mRNAs and microRNAs to specific recipient cells [113]. In this context it has been shown that exosomes affect recipient cells in various ways and elicit cell specific responses [114, 115].

1.3 Scope of this thesis

HDGF is a ubiquitously expressed protein that was reported to be involved in a variety of functions such as cell proliferation, cell migration, RNA processing or ribosome biogenesis. Like the majority of human genes it undergoes alternative splicing, a mechanism which may contribute to the functional diversity of HDGF by the generation of alternative isoforms. Screening the NCBI database revealed the presence of several alternative human HDGF transcripts, generated by alternative splicing. Despite their entry in the NCBI database, these HDGF isoforms have remained mostly unnoticed in the HDGF research field by now. Therefore, one of the main objectives of this thesis was the verification and initial characterisation of two novel HDGF isoforms, termed HDGF-B and HDGF-C.

The two alternative variants exhibit differences in the N-terminal part compared to the well described original HDGF form, now termed HDGF-A in the further course of this thesis. Since most functions and protein interactions of HDGF-A are assigned to the N-terminal hath region, the alteration in this part of the protein by alternative splicing might have huge impact on the function. To characterise these novel HDGF isoforms and to get an impression about their functional role, different assays were performed with respect to known features of HDGF-A. Moreover, a central point was the identification of interaction partners of the novel isoforms, since this might reveal insight into their biological role.

A second important aspect of this thesis was the secretion of HDGF-A. Although numerous studies demonstrated that HDGF can be detected in the surrounding of cells and moreover exogenous HDGF-A realizes extracellular tasks, it is still obscure how HDGF leaves the cell.

With regard to the finding that exosomes are a vehicle of HDGF, the identification of HDGF-A as well as of its alternative isoforms HDGF-B and HDGF-C in exosome
enriched fractions was one aspect. Proteins can be localised within extracellular vesicles or present on the surface and this localisation is essential with regard to potential downstream cell communication processes. Therefore, the localisation in such extracellular vesicles was a further aspect. Since posttranslational dephosphorylation at serine residue 165 in HDGF-A appears to influence secretion of HDGF-A, this mutant variant of HDGF-A was included in the present study.
2. Material and Methods

2.1 Methods

2.1.1 Nucleic acids preparation

2.1.1.1 RNA isolation from cultured cells using Trifast reagent

Total RNA was isolated according to the manufacturer’s guidelines from following cultured cells: HepG2, HeLa, MCF-7, MDA-MB-231 or primary human dermal fibroblasts. Cells were grown on 3.5 cm tissue culture plates. Confluent grown cells were lysed in 1 mL of Trifast reagent and lysates were transferred into 1.5 mL tubes. To achieve dissociation of nucleoprotein complexes, lysates were incubated for 5 min at RT. Next, 200 µL of chloroform were added to the cell lysates followed by incubation for 10 min at room temperature (RT). The samples were centrifuged at 12 000 x g for 5 min to separate the mixture into the lower red phenol-chloroform phase, the interphase and the colourless upper aqueous phase. The aqueous phase, containing RNA, was transferred into a new 1.5 mL tube. RNA was precipitated by adding 0.5 mL of isopropanol and samples were kept for 10 min on ice followed by centrifugation at 12 000 x g for 10 min at 4 °C. The obtained pellet was subsequently washed with 75% (v/v) ethanol (ethanol/H2O) and once more centrifuged at 12 000 x g for 10 min at 4 °C. Finally the pellet was resuspended in 50 µL of RNAse free water. Quality and concentration was determined by measuring the absorbance using the Nanodrop.

2.1.1.2 Synthesis of cDNA by reverse transcription

Thermo Scientific RevertAid First Strand cDNA Synthesis Kit was used to transcribe RNA into complementary DNA (cDNA) following the manufacturer’s guidelines. Therefore, 2 µg of total RNA was mixed with 1 µL of oligo (dT)18 primer (100 µM), 4 µL of the provided Reaction buffer (5x), 1 µL of RiboLock RNase Inhibitor (20 U/µL), 2 µL of dNTP mix (10 mM) and 1 µL of RevertAid M-MuLV RT (200 U/µL). Nuclease-free water was added to a final volume of 20 µL, samples were briefly mixed and centrifuged, followed by an incubation at 42 °C for 45 min. Finally the reaction was terminated by heating at 70°C for 5 min and samples were stored at -20 °C.

2.1.1.3 Polymerase chain reaction

Polymerase chain reaction (PCR) is one of the most important technologies in molecular biology with widespread applications. In general the technology is based on the
amplification of DNA fragments by a thermostable DNA polymerase. *Pfu* DNA polymerases possess 3’ to 5’ exonuclease proofreading activity, which leads to low error rates. Therefore, this enzyme was used for reactions which required high fidelity. PCR reaction mixture (total volume of 50 µL) was set up on ice with following components.

1 ng DNA
1 µL sense primer (100 pmol)
1 µL antisense primer (100 pmol)
1 µL dNTP’s (10mM)
5 µL 10X *Pfu* Buffer with MgSO$_4$
0.5 µL *Pfu*-Polymerase (2.5 U/µL)
H$_2$O up to 50 µL

PCR samples were briefly mixed and spinned down. Then PCR reaction was performed in a thermocycler using the following cycling conditions (table 1).

### Table 1: Cycling conditions for PCR.

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>62</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

### 2.1.1.4 Agarose gelelectrophoresis

Agarose gelelectrophoresis is used for separation of DNA fragments according to their length. To visualise DNA in the gel, fluorescent dyes such as ethidium bromide or less toxic DNA stain G are added to the gel. These dyes bind to DNA, which allows the visualisation of DNA fragments.

To prepare agarose gels, respective amount of agarose was weighed into a specific volume of 1x TAE (tris-acetate-EDTA) buffer to obtain the gel concentration of choice. The solution was heated in a microwave until agarose was dissolved completely. After cooling down to about 60 °C, DNA stain G was added to the agarose solution in a working dilution of 1:20 000, the solution was poured into gel cassettes, and combs were inserted. After polymerization, the comb was removed, and the gel was covered with 1 x TAE buffer. Samples, mixed with Mass Ruler DNA loading Dye (6 x) in a 1:6 ratio, and
Mass Ruler DNA Ladder Mix were loaded into the wells. Electrophoresis was performed at 100 V for approximately 45 min. Finally, gels were illuminated with UV light to detect DNA fragments. To document results, a picture of the gel was captured.

2.1.1.5 Real-time quantitative PCR

Real-time quantitative PCR (qPCR) allows quantification of mRNA expression. It includes the reverse transcription of mRNAs (section 2.1.1.2) followed by quantitative PCR. Because of the high sensitivity, this method allows quantification of rare target transcripts as well as the detection of small changes in expression [116]. To monitor amplification of products during each cycle, the fluorescent dye SYBR Green, which intercalates in double stranded DNA [117], was used. Each reaction set up was prepared as triplicate and contained 5 µL of Power SYBR Green Mastermix (2 x), 1.25 pmol forward primer, 1.25 pmol reverse primer and 10 ng cDNA in a total volume of 10 µL. Reaction was performed with the StepOnePlus™ Real-Time PCR cycler.

In this work relative expression was determined by the comparative 2^{ΔΔCT} method [118]. As reference gene microglobulin was used. Efficiency of each primer pair was determined from standard curves. Therefore, five serial 10 fold dilutions of cDNA were prepared. The obtained CT values were plotted against the cDNA input and efficiencies were calculated from the slope of the obtained standard curves. The PCR programme (table 2) was optimised for each primer pair in such a way that resulting calculated PCR efficiencies were between 95 – 100 %.

Table 2: Real-time quantitative PCR programme.

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>*Primer dependent</td>
<td>30 sec</td>
<td>40</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Melting curve</td>
<td>Default settings</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Annealing temperature:

HDGF A/ HDGF B: 60 °C
HDGF C: 60.5 °C
Microglobulin: 62 °C
2.1.2 Methods for preparing recombinant plasmids

2.1.2.1 Restriction enzyme digestion

Restriction enzymes are able to cleave DNA at specific recognition sites. Wide variety of enzymes with different target sequences is commercially available, allowing diverse cloning strategies. Depending on the used restriction enzyme, restriction digest can result in double stranded DNAs with blunt end or sticky ends, which have either a 3′ or 5′ nucleotide overhang.

Restriction digest was set up in 20 µL containing 2 µL 10 x FastDigest Green Buffer, 1 µg of DNA, and 0.5 U of restriction enzyme. In case of double digest, the second restriction enzyme was added to the same set up. Mixture was incubated for 60 min at 37 °C.

2.1.2.2 Gel extraction from agarose gels

To isolate a specific fragment from agarose gels following agarose gel electrophoresis (section 2.1.1.4), the GeneJet Gel Extraction Kit was used according to the manufacturers’ guidelines. The stained gel was illuminated with blue light in order to visualise the DNA. Desired band was excised with a scalpel and transferred to a pre-weighed 1.5 mL tube. The weight of the gel slice was determined and Binding Buffer was added in a ratio of 1:1 (weight: volume). After incubating the mixture for 10 min at 50 – 60 °C, the solubilised gel solution was transferred to a GeneJet purification column followed by centrifugation with a table-top microcentrifuge at full speed for 1 min at RT. The flow through was discarded and 700 µL of wash buffer was added to the column. After centrifugation at same conditions, the flow through was discarded and centrifugation was repeated to remove residual wash buffer. Finally the column was placed into a new 1.5 mL tube, 50 µL of Elution Buffer was added to the column and the sample was centrifuged under same conditions. Concentration of purified DNA was determined using Nanodrop instrument and the sample was stored at -20 °C.

2.1.2.3 Ligation

DNA ligases catalyze the formation of phosphodiester bonds between two DNA molecules. Therefore, these enzymes can be used to link insert DNA, containing the sequence of interest, into the respective vector DNA.

Ligation reaction set up was prepared in 20 µL total volume containing 2 µL of 10 x ligase buffer, 2 µL of T4 Ligase enzyme and 25 ng vector DNA. Insert DNA were added in a molar ratio of approximately 1:3 (insert : vector). After mixing components briefly, the set up was incubated for 2 h at RT. Reaction was heat inactivated at 65 °C for 10 min.
2.1.2.4 Transformation of competent bacterial cells

Introducing foreign DNA into bacterial cells is termed transformation. This widely used technique allows the amplification of recombinant plasmid DNA. To facilitate this, plasmids usually carry a bacterial origin of replication. In addition, plasmids contain an antibiotic resistance gene to allow selection of positive clones.

200 µL of half thawed chemically competent *E. coli* cells were gently mixed with 5 – 10 ng of plasmid DNA. After incubating the mixture for 10 min on ice, the tube was transferred to a 37 °C heat block for 90 sec (heat shock). The transfection mixture was incubated for 2 min on ice before 1 mL of pre-warmed SOC medium was added. The mixture was incubated in the 37°C shaker for 45 min. Finally the culture was spread on a pre-warmed LB-agar plate. Depending on the antibiotic resistance encoded by the respective plasmids, LB-agar plates containing the respective antibiotic (50 µg/mL ampicillin or 50 µg/mL kanamycin) were selected.

2.1.2.5 Mini-preparations of plasmid DNA

The mini-preparation of plasmid DNA allows the isolation of plasmids from bacteria in small scales and is used to screen for positive clones.

GeneJET Plasmid Miniprep Kit was used according to the manufacturers’ guidelines. 5 mL of LB-medium containing the appropriate selection antibiotic was inoculated with the respective clone and incubated over night at 37 °C under shaking conditions. In parallel, the same clone was transferred to a new LB-agar plate with numbered spots. Next day, the overnight culture was centrifuged at 6 000 x g for 5 min at RT to harvest bacterial cells. The pellet was resuspended in 250 µL of Resuspension solution and transferred to a 1.5 mL tube. To achieve lysis of bacterial cells, the resuspension was mixed with 250 µL of Lysis solution. After 5 min incubation at RT, 350 µL of Neutralization solution was added, and cell debris removed by centrifugation in a table-top microcentrifuge at 12 000 x g for 5 min at RT. The obtained supernatant was transferred to a GeneJET spin column and centrifuged at full speed for 1 min. Flow through was discarded and 500 µL of Wash Solution was added to the column followed by a second centrifugation (1 min, 20 000 x g, RT). The column was centrifuged for a second time to remove residual Wash solutions. Finally the column was placed into 1.5 mL tube, 50 µL of Elution buffer was added to centre of the column and after incubation of 2 min, the column was centrifuged (1 min, 20 000 x g, RT). Purified plasmid DNA was stored at -20 °C.
2.1.2.6 Plasmid purification

Plasmids were purified from bacterial cultures by using NucleoBond Xtra Midi kit (Machery-Nagel) according to the manufacturers’ guidelines. A clone containing the plasmid with the correct insert was transferred to 200 mL of sterile LB-medium. Depending on the antibiotic resistance encoded by the plasmid, the respective antibiotic was added to the liquid culture to a final concentration of 50 µg/mL. This culture was incubated at 37°C over night under shaking conditions (250 rpm).

The next day, the overnight culture was centrifuged at 6000 rpm for 10 min at 4 °C to harvest bacterial cells. While the supernatant was completely removed and discarded, the obtained cell pellet was resuspended in 8 mL of the provided buffer RES (+RNase A). The cell suspension was transferred into a new 50 mL tube. For cell lysis 8 mL of the provided buffer LYS was added and the solution was mixed by inverting the tube several times. After incubation for 5 min at RT, bacterial cell lysate was neutralised by adding 6 mL of Buffer NEU. The resulting precipitate was mixed several times by inverting the tube. Meanwhile, the column and filter were pre-equilibrated with 12 mL of the provided buffer EQU and the precipitate was loaded to the column. After the cell lysate flowed completely through the column, the column was washed with 5 mL of the buffer EQU. Before the column was washed with 5 mL of buffer WASH, the filter unit was discarded. The collected flow through was also discarded and the column with the bound DNA was transferred to a new 50 mL tube. Finally the bound DNA was eluted by adding 5 mL of buffer ELU to the column. To concentrate the plasmid containing eluate, the eluate was mixed with 3.5 mL isopropanol, vortexed, and incubated for 2 min at RT. In the meantime the plunger of a 30 mL syringe was removed and a NucleoBond finalizer was attached to the outlet of the syringe. After filling the syringe with the precipitation mixture, the mixture was slowly pressed through the NucleoBond finalizer. The resulting flow through was discarded and the NucleoBond finalizer was washed with 2 mL 70 % (v/v) ethanol (ethanol/H₂O). Prior to DNA elution, the NucleoBond finalizer was completely dried. Therefore, Nucleobond finalizer was detached from the syringe, the plunger was inserted and after reattaching the Nucleobond finalizer to the syringe, air was pressed through the finalizer several times. Finally the NucleoBond finalizer was attached to 1 mL syringe. The syringe was filled with 800 µL of redissolving buffer TRIS and solution was slowly pressed through the NucleoBond finalizer.

To determine the concentration and quality of the obtained plasmid solution, the Nandrop instrument was used.
2.1.2.7 Sequencing of DNA segments

To verify constructs, sequencing reactions were performed. Reaction mixture was set up with 20 – 50 ng of the plasmid DNA containing the sequence of interest, 2 µL of Big Dye Terminator and 50 pmol primer in a total volume of 11 µL. Reaction was performed in PCR cycler over night as shown in table 3.

Table 3: Sequencing reaction

<table>
<thead>
<tr>
<th>PCR Programme</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>56</td>
<td>15 sec</td>
<td>60</td>
</tr>
<tr>
<td>Elongation</td>
<td>60</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

The next day, 9 µL of distilled H₂O was added to the reaction mixture and sequencing reaction was purified via gel filtration using 96 well spin plates (supplied by the Max Planck Institute for Marine Microbiology). Purified samples were analyzed in Max Planck Institute for Marine Microbiology (Bremen).

2.1.3 Cell culture

All work with mammalian cells was performed under sterile conditions. Only sterile filtered media and solutions were used and the work was performed under a sterile bench. Buffers, solution, and media were pre-warmed to 37 °C before usage. All cells were cultured at 37 °C in a humidified atmosphere of 5 % (v/v) CO₂.

2.1.3.1 Culture of immortalised cells

For this work several immortalised cells were used. Cos7, HepG2, HeLa, MCF-7, MDA-MB-231 and HaCat cells grow as adherent monolayer in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % heat-inactivated fetal calf serum (FCS) and 50 µg/mL gentamicin. For passaging cells, the old medium was aspirated and cells were washed with phosphate buffered saline (PBS). To dissociate cells, trypsin was added to the flask (approximately 1 mL per 10 cm²) and cells were incubated for 3-10 min at 37 °C (exact incubation time varied with used cell line). Detached cells were resuspended in new medium and centrifuged for 5 min at 400 x g. Cell pellets were resuspended in fresh media and distributed onto new plates.
2.1.3.2 Human dermal fibroblast primary culture

The experiments were undertaken with the understanding and written consent of each subject. The study methodologies conformed to the standards set by the Declaration of Helsinki. The Ethics Committee of the Medical Chamber of Bremen (No. 336/12) approved the study.

Skin was derived from reduction surgery of healthy Caucasian patients. After removal the fat-free skin was stored at 4°C in 0.9 % NaCl (saline) until cell isolation. Isolation was performed within 4 h after surgical intervention. First, skin was immersed several times in PBS containing penicillin-streptomycin (50 µg/mL) and cut into smaller pieces. To facilitate separation of dermis and epidermis, skin pieces were incubated in Dispase II solution at 4 °C overnight. Next day dermis was mechanically separated from epidermis by forceps. To achieve single cell population, dermis was minced into smaller pieces with scissors, transferred into collagenase type 2 solution and incubated for 2 h in a 37 °C water bath under shaking conditions. The mixture was filtered through cell strainer (pore size 70 μm), and the filtrate was centrifuged at 400 x g for 5 min at RT. The obtained cell pellet was resuspended in culture medium DMEM/Ham’s F12, supplemented with FCS (10 % v/v), L-glutamine (5 mM), penicillin/streptomycin (100 µg/mL) and cells were seeded into culture dishes. Fibroblasts were cultured at 37 °C in a humidified atmosphere of 5 % (v/v) CO₂. For passaging cells, same protocol as for immortalised cells (section 2.1.3.1) was performed.

2.1.3.3 Cryopreservation of cells

Cells (immortalised cells lines and primary human fibroblasts) which reached confluence were harvested from 10 x 10 cm plates (as described in section 2.1.3.1). The obtained cell pellet was resuspended in freezing medium containing DMEM supplemented with 20 % FCS, 10 % DMSO (1 mL per 10 cm² plate) and collected in freezing vials. Before freezing cells for long time storage in liquid nitrogen, cells were stored for 30 min at 4 °C and then for several days at - 80 °C.

2.1.3.4 Transient transfection of HepG2 and Cos7 cells

Polyethylenimine (PEI) is a stable cationic polymer that can deliver DNA into cells. PEI complexes DNA into positively charged particles that bind to anionic cell surfaces. Such complexes can enter the cell by endocytosis [119].

For transfection cells have ideally reached a density of around 80 % confluence. Prior to transfection, the medium was changed to DMEM supplemented with 2 % FCS (v/v). To transfecct cells on 10 x 10 cm plates, 15 µg of plasmid DNA, containing the gene of
interest, were mixed with 1 mL of NaCl solution (150 mM). For transfection of cells grown on 24 well plates, 1.5 µg of plasmid DNA were added to 100 µL NaCl solution. Cells on 96 well plates were supplemented with 150 ng of plasmid DNA in 10 µL of NaCl solution. PEI stock solution in a dilution of 1/750 (v/v) in 150 mM NaCl were prepared prior to transfection. 25 µL (10 cm plate), 2.5 µL (24 well plate) or 0.25 µL (96 well plate) of PEI stock solution were added to the transfection mixture. After vortexing for 10 sec, the mixture was incubated for 10 min at RT. As next, transfection mixture was added to the cells and cells were incubated for approximately 4 h at 37 °C with 5 % (v/v) CO₂ in the incubator before the medium was changed to serum-free DMEM. In case of co-transfection with two plasmids encoding two different constructs, half of the indicated amount per plasmid was used, so that the sum of both corresponded to that of a single transfection.

2.1.4 Immunofluorescence analysis

Immunofluorescence is a microscope based technique that allows visualisation of intracellular proteins and structures. It utilizes fluorescent-labelled antibodies to detect specific target proteins. Cells, grown on coverslips in a 24 well plate, were washed two times with PBS. In order to fix cells, cells were incubated in 4 % PFA solution (300 µL/ well). After removing the PFA solution from the cells, cells were incubated in ice-cold methanol (100 %) (250 µL/ well) for 2 min at RT. Fixed and permeabilised cells were washed two times with PBS containing 2 % BSA (w/v). To block the non-specific binding of antibody to the specimen, DMEM containing 10 % FCS (v/v) was used. Blocking lasted 30 min (at RT). Then primary antibody was added to the cells and cells were incubated for 1.5 h at RT. The used antibody concentration varied from type to type (concentrations are given in section 2.2.10). After this incubation cells were again washed with PBS (2 % BSA) before secondary antibody was added and cells were incubated for 1.5 h at RT in the dark. The secondary antibody (donkey anti-rabbit antibody Cy3 and donkey anti-mouse antibody Cy5 conjugate) was diluted 1/500 (v/v) in PBS / 2 % BSA (w/v). Finally cells were washed twice with PBS. To preserve specimens, the cells were embedded in Fluoro-Gel II. Images were captured with an Apotome fluorescence imager.

2.1.5 Functional assays
2.1.5.1 BrdU proliferation assay

For proliferation studies Cos-7 cells were plated at a density of 2500 cells per well in a 96 well plate. Cells were transiently transfected with plasmids coding for untagged HDGF-A,
HDGF-B, HDGF-C or with the respective empty vector as described in section 2.1.3.4. For one experiment 10 replicates per condition were examined. 4 h after transfection medium was changed to serum free media followed by further incubation for 20 h at 37°C with 5% CO₂. To measure proliferation rates, the Cell Proliferation ELISA kit (Roche) was used according to the manufacturers’ instructions. Bromodeoxyuridine (BrdU) was added to the media followed by 6 h incubation at 37°C with 5% CO₂. After this labelling period, the labelling medium was removed and cells were fixed by adding 100 µL of FixDenat reagent to the cells for 30 min at RT. Then FixDenat solution was removed completely and cells were covered with anti-BrdU-POD-working solution for 1.5 h at RT. Finally cells were washed 5 times with PS to remove residual antibody. To measure the intensity of cell proliferation, 100 µL of substrate solution was added to each well with a multichannel pipette. After 10 min of incubation at RT the reaction was stopped by adding 25 µL 2 M H₂SO₄. The absorbance at 450 nm was recorded.

2.1.5.2 Chromatin-binding assay

In order to determine association to chromatin of the target protein, the chromatin-binding assay was performed as described by Thakar et al. [49] with some minor modifications. Therefore, HepG2 on 10 x 10 cm plates were transiently transfected as described in section 2.1.3.4. 24 h after transfection, cells were lysed for 10 min on ice in 1 mL of ice cold CSKI buffer. Then cells were scraped using a cell scraper and corresponding lysates were transferred to a 1.5 mL tube. An aliquot of 100 µL was removed from this fraction and mixed with the same volume of RIPA buffer. This fraction represented the total volume (T). The remaining cell lysate was divided into two equal portions which were centrifuged at 450 x g at 4°C for 3 min. The supernatants were collected and pooled. To obtain the S1 fraction, the pooled supernatants were centrifuged a further time for 10 min at 12 000 x g. The remaining pellets, obtained after 450 x g centrifugation step, contained chromatin-bound, nuclear matrix bound, and insoluble proteins. To remove possible contaminations, pellets were washed with CSKI buffer, followed by a second centrifugation at 450 x g at 4°C for 3 min. CSK I buffer was completely removed and one of the pellets was resuspended in RIPA buffer to get the P1 fraction. The second pellet was resuspended in 200 µL of CSK II buffer und the resuspension was treated with 6 U/µL DNase for 30 min at RT. NH₄SO₄ was added to a final concentration of 250 mM followed by incubation for 10 min at RT. The sample was then centrifuged at 1 200 x g for 6 min at 4°C and the supernatant containing DNase-released chromatin-associated proteins (S2) was transferred to a new 1.5 mL tube. The pellet, which consisted of insoluble, cytoskeletal, and nuclear matrix proteins, was washed with CSKII buffer and centrifugation was repeated at 1 200 x g for 6 min at 4°C.
The supernatant was completely discarded and the obtained P2 pellet was resuspended in 100 \( \mu L \) RIPA buffer. Finally protein concentration of each fraction was determined by BCA assay (section 2.1.9.3) and 20 \( \mu g \) of each fraction were mixed with 2 x sample buffer supplemented with DTT, boiled for 5 min at 95 °C and subjected to SDS PAGE followed by Western blot analysis.

2.1.5.3 Bimolecular fluorescence complementation (BiFC) assay

Cos7 cells were grown on cover slips in 24 well plates. Transient transfection was carried out as described under section 2.1.3.4. The used plasmids encoded the HDGF isoforms (-A, -B, -C) fused to the N-terminal part (aa 1-172) or the C-terminal part (aa 173-238) of enhanced yellow fluorescent protein (EYFP). 24 h after transfection, cells were transferred from 37 °C incubation conditions to 30 °C for 30 min to promote fluorophore maturation. Controls were incubated for 90 min under the same conditions. Prior to fixation with 4 % PFA solution for 10 min at RT, cells were washed twice with PBS. Fixed cells on cover slips were embedded in Fluoro-Gel II containing DAPI (4’,6-diamidino-2-phenylindoledihydrochloride) to stain the nuclei. Stained cells were imaged with a fluorescent microscope (Olympus).

2.1.6 Inhibitor studies

In the case of inhibitor studies, transfected HepG2 cells were exposed to the inhibitor 5-(N, N-dimethyl) amiloride (DMA) or Monensin (Mon). Transient transfection was performed as described under section 2.1.3.4. 3 h after transfection, the transfection mixture were aspirated and medium containing the following inhibitors were added: DMA in a final concentration of 15 nM (stock solution 15 \( \mu M \) in DMSO); Monensin 100 \( \mu M \) (stock solution 100 mM in EtOH). Cells were incubated for further 24 h or 40 h in the incubator (37 °C, 5 % \( CO_2 \)) before supernatant and cell lysate were collected for subsequent analysis. Collected supernatants were concentrated by acetone precipitation (section 2.1.9.2).

2.1.7 LDH assay

Lactate dehydrogenase (LDH) assay is a method to quantify the cell viability. LDH is a ubiquitously expressed cytoplasmic protein that catalyzes the redox reaction of pyruvate to lactate in the presence of NADH. After the loss of membrane integrity due to cell death, LDH is passively released like other cytoplasmic proteins in the surrounding medium. Therefore, the measurement of extracellular LDH activity can be used as indicator for cell death [120].
The activity of LDH can be determined by measuring the decrease of NADH photometrically at 340 nm. The respective protocol was adapted from Dringen et al. [121] with some modifications. 10 µL of the incubation medium was mixed with 170 µL LDH buffer on a 96-well microtitre plate. Directly after addition of 180 µL fresh prepared LDH reaction buffer the decrease of absorbance at 340 nm was measured with a microtitre plate reader (Sunrise, Tecan, Austria) for 10 min in 30 sec intervals. To obtain a percental value, the measured activity was compared with 100 % LDH activity after total lysis. For total lysis Triton X-100 was added to the incubation medium to a final concentration of 1 % (w/v) and cells were incubated for 30 min at RT, before an aliquot was taken for the photometrical measurement. This 100 % LDH activity corresponds to 0 % cell viability. Measurement was performed in triplicates; values are given in % of total LDH ± standard deviation.

2.1.8 Exosomes

2.1.8.1 Isolation and sample preparation

To isolate exosomes from cell culture supernatants, the standard ultracentrifugation protocol for the isolation and enrichment of exosomal vesicles from Théry et al. [99] was used. The protocol consisted of several different centrifugation steps as depicted in figure 5.

Figure 5: Centrifugation protocol for exosome isolation. Exosomes were isolated by a series of centrifugation steps. During each step an aliquot of the supernatant (SN1-5) was collected. The final pellet (exosome-enriched fraction, Ex) was resuspended in PBS.

Depending on the initial issue, exosomes were isolated from transiently transfected HepG2 cells or several non-transfected cell lines. Since FCS contains exosomes [99], cells were incubated in medium without FCS in order to avoid any contaminations by FCS derived exosomes. For one exosome preparation cells were seeded on 10 cm² cell plates and after cells reached approximately 80 % confluency, medium was changed to serum free medium. In the case of transfected cells, medium was changed 4 h after the transient transfection of cells (section 2.1.3.4). Cells were incubated 35-40 h at 37 °C
and 5% CO₂. For each exosome isolation the medium from seven 10 cm² plates were collected (approximately 70 mL of conditioned medium). The collected supernatant was directly subjected to centrifugation at 300 x g for 10 min at 4 °C to remove dead cells and large cell debris. The obtained supernatant was transferred to a new tube and the next centrifugation was performed at 2 000 x g, for 20 min at 4 °C. Again the obtained pellet was discarded. For the following centrifugation steps the ultracentrifuge (Ultracentrifuge Le-80K, Ti45 rotor) was used. The supernatant was centrifuged at 15 000 x g at 4 °C for 30 min and the resulting supernatant was transferred to new tubes. In order to separate exosomes from the supernatant, the supernatant was centrifuged at 120 000 x g at 4 °C for at least 2 h. The pellet was resuspended in 1 mL of PBS and pellet suspensions of the same cell type were pooled. To eliminate contaminating proteins, the tube was completely filled with PBS and centrifuged again (120 000 x g, 4 °C, at least 2 h). The final pellet was resuspended in 100 μL of PBS. For SDS PAGE 100 μL of 2x SDS Sample buffer + DTT were added and the mix was boiled for 5 min at 95 °C. During each step an aliquot of 250 μL was taken from the supernatants as illustrated in figure 5. In order to concentrate collected supernatants they were acetone precipitated as described in section 2.1.9.2.

In order to deplete supernatants from vesicles, an aliquot of 1 mL was collected from SN5 and were ultracentrifuged at 112 000 x g for 24 h (Ultracentrifuge Optima MaxE, TLA 55 rotor). The obtained vesicle-depleted supernatant (termed SN5*) was also acetone precipitated (section 2.1.9.2).

### 2.1.8.2 Dynamic light scattering measurement of exosomes

To characterise the isolated exosome fraction, the hydrodynamic diameter of the vesicles was determined by dynamic light scattering in a Beckman Coulter (Krefeld, Germany) Delsa™ Nano C particle analyser at scattering angles of 165 and 15° at 25 °C. Fresh prepared exosomes were diluted in filtrated PBS. The sample temperature was allowed to equilibrate for 30 min before each measurement. The light scattering was recorded for 900 s with six replicate measurements.

### 2.1.8.3 Pull down assay of exosome fraction

The pull down assay was applied immediately after the exosome isolation. An aliquot of the exosome enriched fraction was disrupted by 1% Triton X-100 (v/v) for 30 min at 4 °C. Equal volumes of disrupted and intact exosomes (20 μL) were then subjected to trypsin digestion at 37 °C for 30 min. Trypsin (stock solution 150 μg/ml) was used in a final concentration of 15 μg/mL. After the trypsin digestion, the samples were mixed with
an equal volume of 2 x sample buffer. After boiling samples at 95 °C for 5 min samples were stored at -20 °C until they were subjected to Western blot analysis.

2.1.9 Sample preparation and analysis

2.1.9.1 Preparation of total cell lysates

Confluent grown cells on 10 x 10 cm culture dishes cold were washed twice with PBS twice. To lyse cells, 1 mL of cold TNE lysis buffer containing freshly added protease inhibitor (Complete Mini, EDTA free) was added to each plate. After incubation for 10 min on ice, the cells were scraped from the plate and the cell homogenate was transferred to a new reaction tube. A second incubation on ice followed for 10 min. Finally the homogenate was centrifuged at 20 000 x g for 20 min at 4 °C and the subsequent supernatant was transferred into a new tube while the pellet was discarded. For SDS PAGE an aliquot was taken, mixed with an equal volume of 2 x sample buffer, and boiled for 5 min at 95 °C. The samples were stored at -20 °C until they were subjected to SDS PAGE gels.

2.1.9.2 Acetone precipitation

In order to concentrate proteins from solutions, they were acetone precipitated. The collected samples were mixed with acetone in a ratio of 1:4 (sample: acetone) and stored at -20 °C for at least 12 h. Then, the samples were centrifuged at 20 000 x g at 4 °C for 20 min. The supernatant was discarded while the obtained pellet was resuspended in 40 µL of 2 x sample buffer. After boiling the samples at 95 °C for 5 min the samples were either stored at -20 °C or directly subjected to SDS PAGE analysis (section 2.1.9.6).

2.1.9.3 BCA assay

In order to determine the protein concentration, the Pierce BCA Protein Assay Kit was used according to the manufacturer’s guidelines. The principle of the bicinchoninic acid assay (BCA) relies on a colour reaction which is proportional to the amount of protein present [122].

At the beginning a set of protein standards containing albumin in known concentration were prepared as serial dilution. Then 12.5 µL of each standard or unknown sample replicate was pipetted into a microplate well (working range = 20-2000 µg/mL). To prepare the working reagent, 50 parts of solution A was mixed with 1 part of solution B. To each well 100 µL of the working reagent was added, the plate was covered with a sheet of parafilm and, after mixing the plate on a shaker, the plate was incubated for 30 min at 37 °C. Finally absorbance was recorded with a plate reader at 560 nm.
2.1.9.4 Immunoprecipitation

This technique allows the precipitation of proteins out of a solution by using a protein specific antibody. The resulting antibody/antigen complex can be isolated by the use of protein A sepharose beads. While precipitating the target protein, possible interaction partners can be co-precipitated.

Samples of interest, in this case, total cell lysates were prepared from 10 x 10 cm culture dishes of confluent grown HepG2 cells as described under section 2.1.9.1. Protein A sepharose beads were pre-equilibrated by washing beads 3 times with TNE lysis buffer. During each washing step, samples were centrifuged at 1,200 x g at RT for 15 sec in order to sediment the protein A beads. To prevent unspecific contaminations, the cleared lysates were pre-cleared. Therefore, 1 mL of cell lysate was mixed with 100 µL of pre-equilibrated protein A sepharose beads and incubated for 2 h under rotary agitations at 4 °C. Beads were centrifuged at 1,200 x g for 15 sec at RT and the pre-cleared supernatant was collected for the precipitation. The pre-cleared control fraction for Western blot analysis was prepared as follows: The beads were washed five times with 1 mL of TNE lysis buffer. Finally supernatant was completely removed and 100 µL of 2 x sample buffer was added. Beads were boiled for 10 min at 95 °C. Finally the sample was centrifuged (1,200 x g, 30 sec, RT) and the supernatant was collected and stored at -20 °C.

To the pre-cleared cell lysates 50 µg of isoform specific antibodies anti-HDGF-A, anti-HDGF-B, anti-HDGF-C or 20 µg of goat anti pan-HDGF was added. After an incubation of 2 h on ice 100 µL of pre-equilibrated protein A beads were added. The lysate/-beads mixture was incubated over night at 4 °C under rotary agitation. The next day, the protein A sepharose beads were treated as described for the pre-cleared control fraction.

2.1.9.5 StrepTactin co-precipitation

In general a similar protocol as described for immunoprecipitation was used (section 2.1.9.4). HepG2 cells on 10 x 10 cm culture dishes, transiently transfected with plasmids encoding Strep-tagged proteins were lysed and cleared lysates were mixed with 50 µL of pre-equilibrated StrepTactin beads. The lysate/-beads mixture was incubated over night at 4 °C under rotary agitation. The washing and elution procedure resembled that protocol for immunoprecipitation.

In case of RNAse digest experiments, the protocol from Bremer et al. was used [123]. Protein lysates from HDGF-A/B/C-Strep expressing HepG2 cells were pre-incubated with or without RNAse A (25 µg/ mL) at 4 °C over night followed by StrepTactin precipitation as described before.
2.1.9.6 SDS PAGE

SDS PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) was performed according to Laemmli with slight modifications [124]. This technique separates proteins mainly according to their molecular weights. The anionic detergent sodium dodecyl sulfate (SDS) binds to the polypeptide chain, causes the denaturation of proteins and imparts an even distributed charge to the protein. The addition of dithiothreitol (DTT), a strong reducing agent, causes the disruption of disulfide bonds.

First, the gels were prepared. Glass plates were cleaned with 70 % (v/v) (ethanol/H₂O) followed by assembling the gel cassettes according to the manufacturers’ guidelines. To prepare 12 % gels the ingredients, listed in table 4, were mixed. Obtained solution was transferred to the gel cassette and the gel was overlaid with water-saturated butanol. After the polymerisation of the separation gel, the butanol layer was removed and gels were rinsed with water to remove residual butanol. Finally the stacking gel was prepared on top of the separation gel according to the ingredient list in table 1 and appropriate comb was inserted. After polymerisation the comb was removed, the electrophoresis unit was assembled, and the electrophoresis chamber was filled with SDS PAGE running buffer.

Samples were mixed with 2 x sample buffer and were directly boiled for 5 min at 95 °C before loading. When samples were analyzed under non-reducing conditions, 2 x sample buffer without DTT was used. This was the case for samples that were immunodetected with anti-CD63 antibody.

20 µL of the prepared samples as well as 5 µL of pre-stained protein marker (Thermo Scientific GmbH) were loaded on the gels. An electric current of 15 mA was applied on the gel until the running front reached the end of the gel.

Table 4: Used ingredients for SDS PAGE gel.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>4 % Stacking gel</th>
<th>12 % Separation gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide, 30 %</td>
<td>333 µL</td>
<td>1.98 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.52 mL</td>
<td>1.77 mL</td>
</tr>
<tr>
<td>4x Stacking/Separation gel buffer</td>
<td>625 µL</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.5 µL</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>12.5 µL</td>
<td>25 µL</td>
</tr>
</tbody>
</table>

If no subsequent Western blot analysis was performed, the gel was stained with PAGE Blue Protein staining solution. PAGE Blue contains the dye Coomassie Brilliant Blue, which binds non-specifically to proteins primarily through basic amino acids. In order to stain the polyacrylamidgel, it was briefly washed with deionised water and then incubated...
in PAGE Blue for 45 min at RT on a shaker. Before scanning the gel, it was de-stained with deionised water to obtain a clear background.

**2.1.9.7 Western blotting and immunodetection**

Western Blotting was performed subsequent to SDS PAGE (section 2.1.9.6). For this purpose the polyacrylamide gel containing the separated proteins, was released from the glass plates and was transferred into blotting buffer for 10 min on a shaker at RT. In the meantime the PVDF membrane was pre-wetted in methanol, then directly washed in distilled water for 2 min and finally equilibrated in blotting buffer for 10 min. For the transfer the gel was placed on one filter pad and two filter papers, each pre-equilibrated in blotting buffer. The PVDF membrane was placed on top of the gel, followed by two filter papers and one filter pad. The transfer was performed for 1 h at 100 V under cooling conditions.

After the transfer the membranes were briefly washed with Tris-buffered Saline + 0.15 % Tween (v/v) (TBST) to remove gel pieces. To prevent unspecific binding, the membrane was blocked in blocking buffer containing TBST + 3 % BSA (w/v) for 1 h at RT on a shaker. After washing with TBST, the membrane was placed on parafilm. For immunodetection 1 mL of primary antibody, diluted in blocking buffer, was added and the membrane was covered with a second sheet of parafilm and was incubated over night at 4 °C. The used concentrations of each primary antibody are listed in section 2.2.10. After several washes with TBST at RT on the shaker for at least 45 min, the membrane was incubated with the corresponding secondary antibody, diluted in blocking buffer, for 1.5 h at RT on a shaker. Prior to detection membranes were washed 6 times with TBST for 1 h at RT.

Membranes were incubated either in Pierce ECL Western Blotting Substrate or Select ECL Western Blotting Substrate. Both solutions contained substrate for the horseradish-peroxidase (POD), an enzyme which is linked to the secondary antibody. The subsequent reaction produces chemiluminescence, which can be captured by the use of X-ray films.

In the case of Pierce ECL Western Blotting substrate, 500 µL of solution A and 500 µL of solution B were mixed. In the case of Select ECL, which was used for weaker signals, 900 µL of TBS-T, 50 µL of solution A and 50 µL of solution B were mixed with each other. The mixture was placed on a sheet of parafilm and the membrane was placed with the protein side into the detection solution for 5 min. Afterwards the membrane was sealed into a plastic foil, placed in a cassette and covered with X-ray film for a specific time period. The film was developed in the developer machine (Kodak, Stuttgart).
2.1.9.8 MALDI TOF-MS

Samples for MALDI TOF-MS were separated by SDS PAGE electrophoresis and Coomassie Blue-stained protein bands were excised from the gel. The gel trypsin digestion was performed as described by Shevchenko et al. [125]. Briefly, gel pieces were dehydrated in acetonitril followed by drying in a speedvac. Dried gel pieces were transferred in 50 mM NH₄HCO₃ containing 10 mM DTT for 1 h at 56 °C to reduce proteins. After cooling to RT, excess solution was removed and gel pieces were covered by 55 mM iodoacetamide in 50 mM NH₄HCO₃ for 45 min at RT in the dark. The gel pieces were washed with 100 µL of 50 mM NH₄HCO₃ for 10 min. Then, gel pieces were dehydrated with acetonitril for 10 min at RT, followed by a rehydration step with 50 mM NH₄HCO₃ and a further dehydration step with acetonitril. The excess solution was removed and gel pieces were completely dried in the speedvac. After drying the gel pieces were covered with digestion buffer containing 25 mM NH₄HCO₃, 5 mM CaCl₂, and 12.5 ng/µL of trypsin for 45 min at 4 °C. Excess trypsin solution was removed and 5-10 µL of the digestion buffer without trypsin was added followed by incubation overnight at 37 °C. Next day gel pieces were washed for 20 min in 20 mM NH₄HCO₃ followed by peptide extraction with three changes of 5 % formic acid in 50 % acetonitril (20 min incubation for each change). The obtained gel pieces were finally dried.

For further analysis the lyophilised peptides were redisolved in 10 µL millipore water and sonicated for 10 min. 1 µL of the undiluted peptide solution as well as 1 µL of a 1:10 (v/v) dilution was spotted on a MTP 384 well ground steel target plate and mixed with MALDI matrix, which contained α-cyano-4-hydroxycinnamic acid dissolved in 50 % acetonitril and 0.2 % TFA at a concentration of 7 mg/mL.

MALDI-TOF was performed by Dr. Janina Oetjen. After crystallisation, mass spectra were acquired in the positive reflector mode on an autoflex speed MALDI-TOF mass spectrometer using flexControl software version 3.4 (Bruker Daltonics). Each spectrum is the sum of 500-1000 laser shots using a Smartbeam™ laser (Bruker Daltonics) using the medium laser focus, a repetition rate of 1000 Hz and the random walk option set on. The spectra were acquired in the mass range of 700-4000 m/z and a deflection of 500 m/z. The external calibration was performed using the monoisotopic peaks of Peptide Calibration Standard II (Bruker Daltonics) consisting of Bradykinin 1-7 [M+H⁺, 757.40], Angiotensin II [M+H⁺, 1046.54], Angiotensin I [M+H⁺, 1296.68], Substance P [M+H⁺, 1347.74], Bombesin [M+H⁺, 1619.82], Renin Substrate [M+H⁺, 1758.93], ACTH clip 1-17 [M+H⁺, 2093.09], ACTH clip 18-39 [M+H⁺, 2465.20], Somatostatin 28 [M+H⁺, 3147.47]. The Cubic Enhanced calibration mode (Bruker Daltonics) was applied.
MALDI-TOF MS data processing and protein identification

The peptide mass fingerprints for protein identification were used and MALDI-TOF mass spectra were processed using the flexAnalysis software version 3.4 (Bruker Daltonics). The Bruker Snap algorithm was used for peak detection with a signal to noise threshold of 6. Maximal 100 peaks in the mass range of 800-4000 were detected. The resulting mass list was searched against the online version of the Mascot peptide mass fingerprint database (Matrix Science, London, UK) using the following settings. Trypsin was selected as enzyme allowing up to 1 missed cleavage. The search was performed against the SwissProt database 2015_3 selecting the protonated ion species and monoisotopic masses. The database entries were reduced to human sequences from HepG2 cells. Propionamid modified cysteine residues and methionine oxidation were selected as variable modifications. The peptide mass tolerance was set between 60-100 ppm.
## Material and Methods

### 2.2 Material

#### 2.2.1 Chemicals

<table>
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<th>Chemical</th>
<th>Company</th>
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<td>1-Butanol</td>
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<tr>
<td>HEPES</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Iodacetamide</td>
<td>Sigma Aldrich, St Louis</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Sigma Aldrich, St Louis</td>
</tr>
<tr>
<td>IPTG (isopropyl-1-thio D galactopyranoside)</td>
<td>Sigma Aldrich, St Louis</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>Sigma Aldrich, St Louis</td>
</tr>
<tr>
<td>MassRuler DNA Loading Dye</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Methanol</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
</tbody>
</table>
### Chemicals and Companies

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinamide dinucleotide (disodium salt) (NADH)</td>
<td>Applichem GmbH, Darmstadt</td>
</tr>
<tr>
<td>NP-40</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Page Blue Protein Staining Solution</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>PeqGOLD TriFast</td>
<td>PEQLAB, Erlangen</td>
</tr>
<tr>
<td>PfU Polymerase</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES)</td>
<td>Roth GmbH, Karlsruhe</td>
</tr>
<tr>
<td>Polyethyleneimine (PEI)</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Power SYBR Green Master Mix</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Protease Inhibitor (Complete Mini, EDTA free)</td>
<td>Roche, Mannheim</td>
</tr>
<tr>
<td>Protein A Sepharose Beads</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>PAGE Blue Protein staining solution</td>
<td>Thermo Fisher Scientific, Waltham</td>
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<tr>
<td>Sodium Chloride</td>
<td>Sigma Aldrich, St. Louis</td>
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<tr>
<td>Sodium dodecyl sulfate</td>
<td>SERVA Electrophoresis GmbH, Heidelberg</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Sodium pyruvat</td>
<td>Merck KGaA, Darmstadt</td>
</tr>
<tr>
<td>StrepTactin sepharose beads</td>
<td>IBA GmbH, Göttingen</td>
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<tr>
<td>Sucrose</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Tetramethylethylendiamin</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Trifluoroacetic acid (TFA)</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Trizma Base (Tris/Cl)</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>USB corporation, Cleveland</td>
</tr>
<tr>
<td>Trypsin</td>
<td>PAA Laboratories GmbH, Pasching</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma Aldrich, St. Louis</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Sigma Aldrich, St. Louis</td>
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### 2.2.2 Buffer and solutions

<table>
<thead>
<tr>
<th>Buffer name</th>
<th>Components</th>
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| Blocking Solution | 10 mM Tris/Cl  
150 mM NaCl  
1.5 % (v/v) Tween  
3 % (w/v) BSA |
| CSK I buffer | 10 mM PIPES  
100 mM NaCl  
1 mM EDTA  
300 mM Sucrose  
1 mM MgCl₂  
1 mM DTT | pH 6.8 |
### Material and Methods

#### Buffer name | Components |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CSK II buffer</strong></td>
<td>10 mM PIPES, 50 mM NaCl, 300 mM Sucrose, 6 mM MgCl₂, 1 mM DTT pH 6.8</td>
</tr>
<tr>
<td><strong>LDH Buffer</strong></td>
<td>200 mM NaCl, 80 mM Tris/Cl pH 7.2</td>
</tr>
<tr>
<td><strong>LDH reaction mix</strong></td>
<td>LDH buffer, 10 mM NADH, 16 mM Sodium pyruvate</td>
</tr>
<tr>
<td><strong>PBS (Phosphate buffered saline)</strong></td>
<td>37 mM NaCl, 2.7 mM Na₂HPO₄, 1.5 mM K₂HPO₄</td>
</tr>
<tr>
<td><strong>RIPA buffer</strong></td>
<td>150 mM Tris/Cl, 150 mM NaCl, 0.5 % (w/v) Sodium deoxycholate, 0.1 % (w/v) SDS, 1 % (v/v) NP-40 pH 8.0</td>
</tr>
<tr>
<td><strong>SDS- PAGE running buffer (10x)</strong></td>
<td>192 mM Glycine, 25 mM Tris/Cl, 0.1 % (w/v) SDS</td>
</tr>
<tr>
<td><strong>SDS-PAGE sample buffer (2x)</strong></td>
<td>10 % (v/v) Glycerol, 4 % (w/v) SDS, 50 mM DTT, 0.02 % (w/v) Bromphenolblue, 125 mM Tris/Cl</td>
</tr>
<tr>
<td><strong>Stacking Buffer (4x)</strong></td>
<td>0.1 % (w/v) SDS, 125 mM Tris/Cl pH 8.8</td>
</tr>
<tr>
<td><strong>Stripping solution for Western blot</strong></td>
<td>100 mM Glycine, pH 3.0; adjusted with HCl</td>
</tr>
<tr>
<td><strong>TAE Buffer (50 x) (Tris-acetate-EDTA)</strong></td>
<td>2.5 mM Tris acetate, 50 mM EDTA pH 8.3</td>
</tr>
<tr>
<td><strong>TBS-T (Tris buffered saline + 1.5 % Tween) (10x)</strong></td>
<td>10 mM Tris/Cl, 150 mM NaCl, 1.5 % (v/v) Tween pH 7.4</td>
</tr>
<tr>
<td><strong>TNE lysis buffer</strong></td>
<td>20 mM Tris/Cl, 150 mM NaCl, 5 mM EDTA, 1 % (v/v) NP-40 pH 7.4</td>
</tr>
<tr>
<td><strong>Western blotting buffer</strong></td>
<td>25 mM Tris/Cl, 192 mM Glycine, 20 % (v/v) Methanol</td>
</tr>
</tbody>
</table>

#### 2.2.3 Cell lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cos7</td>
<td>Kidney fibroblasts from the African green monkey <em>Cercopithecus aethiops</em> [126]</td>
<td>AG Kelm</td>
<td></td>
</tr>
<tr>
<td>HaCat</td>
<td>Immortal human keratinocyte cell line (<em>Homo sapiens</em>) [127]</td>
<td>AG Kelm</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical cancer cells [128]</td>
<td>AG Kelm</td>
<td></td>
</tr>
</tbody>
</table>
### Material and Methods

#### Name | Description | Reference | Source
---|---|---|---
HepG2 | Human cell line derived from a liver tissue with differentiated hepatocellular carcinoma (*Homo sapiens*) | [129] | AG Kelm
MCF-7 | Human breast adenocarcinoma cell line | [130] | Prof. Dr. Jörn Bullerdiek, Centre for Human Genetics, Bremen
MDA-MB-231 | Human breast adenocarcinoma, mammary gland, | [131] | Prof. Dr. Martin Götte, Department of Gynecology and Obstetrics, University Medical Centre Münster

#### 2.2.4 Bacteria strains

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xl1-Blue</td>
<td>Chemically competent <em>E. coli</em> for all cloning and DNA-related work</td>
<td>StrataGene, Santa Clara</td>
</tr>
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#### 2.2.5 Media and solutions for cell culture

<table>
<thead>
<tr>
<th>Media</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture freezing medium</td>
<td>10% (v/v) DMSO 20% (v/v) FCS In DMEM</td>
</tr>
<tr>
<td>Collagenase type 2 solution</td>
<td>2.5 mg/mL collagenase (Sigma Aldrich, St. Louis) 25 mM Hepes, pH 7:0 in Hanks´ Balanced salt solution (sterile filtrated)</td>
</tr>
<tr>
<td>Dispase II solution</td>
<td>2 U/mL Dispase II (Roche, Mannheim) in PBS</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle's Media (DMEM)</td>
<td>13.37 g/L DMEM 3.70 g/L NaHCO₃</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle's Media (DMEM)/Ham’s F12</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>LB agar</td>
<td>15 g/L Tryptone 5 g/L Yeast extract 5 g/L NaCl 15 g/L Agar</td>
</tr>
<tr>
<td>LB medium</td>
<td>15 g/L Tryptone 5 g/L Yeast extract 5 g/L NaCl</td>
</tr>
<tr>
<td>SOC medium</td>
<td>20 g/L tryptone 5 g/L yeast extract 0.5 g/L NaCl 2.5 mM KCl</td>
</tr>
</tbody>
</table>
2.2.6 Primer

Primers were synthesised by Eurofins MWG Biotech (Ebersberg).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sense HDGF-A (BamH1)</td>
<td>CGGGATCCATGTCGCGATCCAACCGGCAG</td>
</tr>
<tr>
<td>sense HDGF-B (BamH1)</td>
<td>CGGGATCCATGCCGCCGAAGGTTGGC</td>
</tr>
<tr>
<td>sense HDGF-C(BamH1)</td>
<td>CGGGATCCATGGAGCAGAGGGCAGGC</td>
</tr>
<tr>
<td>anti-sense HDGF A/B/C primer (EcoRI)</td>
<td>CGGAATTCAGGCTCTCATGATC</td>
</tr>
<tr>
<td>anti-sense HDGF A/B/C primer (HindIII)</td>
<td>CGGAATTCCAGGCTCTCATGATC</td>
</tr>
<tr>
<td>sense-EGFP (EcoRI)</td>
<td>CGGAATTCGATGATTGAACAAGATGGATTG</td>
</tr>
<tr>
<td>anti-sense- EGFP(BamH1)</td>
<td>CGGAATTCGATGATTGAACAAGATGGATTG</td>
</tr>
<tr>
<td>sense primer EYFP (EcoRI)</td>
<td>CGGGATCCATGTCGCGATCCAACCGGCAG</td>
</tr>
<tr>
<td>anti-sense primer HDGF (BamHI)</td>
<td>CGGGATCCATGTCGCGATCCAACCGGCAG</td>
</tr>
<tr>
<td>Real time qPCR Primer A (sense)</td>
<td>ACCGGCAGAAAGGATACAA</td>
</tr>
<tr>
<td>Real-time qPCR Primer B (sense)</td>
<td>TTGTGCGCTCAACTCCTTGG</td>
</tr>
<tr>
<td>Real-time qPCR Primer C (sense)</td>
<td>CGGGAAACCGTGATACAGAC</td>
</tr>
<tr>
<td>Real-time qPCR Primer A/B/C (antisense)</td>
<td>TTGGTATTTGGCTGTTGGA</td>
</tr>
<tr>
<td>Real-time qPCR Microglobulin sense</td>
<td>TTTACTCAGTCATCCAGCAGA</td>
</tr>
<tr>
<td>Real-time qPCR Microglobulin anti-sense</td>
<td>CGGCAGGCATACCATCTTT</td>
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2.2.7 Plasmid vectors

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDGF-A (untagged)</td>
<td>Human HDGF-A sequence was cloned into EcoRI / BamHI digested pcDNA3Amp vector</td>
<td>[132]</td>
</tr>
<tr>
<td>HDGF-A-Strep</td>
<td>Human HDGF-A sequence was cloned into BamHI / EcoRI pcDNA3 Amp Strep-tag vector</td>
<td>[132]</td>
</tr>
<tr>
<td>HDGF-A_CYEFP</td>
<td>CEYFP (aa 173-238 of EYFP) fragment was cloned using EcoRI and XhoI restriction sites of pcDNA3 Amp followed by in frame cloning of the HDGF-A using HindIII and BamHI restriction</td>
<td></td>
</tr>
<tr>
<td>HDGF-A_NYEFP</td>
<td>NEYFP (aa 1-172 of EYFP) fragment was cloned using EcoRI and XhoI restriction sites of pcDNA3 Amp followed by in frame cloning of the HDGF-A using HindIII and BamHI restriction</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Description</td>
<td>Source/Reference</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>HDGF-B (untagged)</td>
<td>Human HDGF-B sequence was cloned into EcoRI / BamHI digested pcDNA3Amp vector</td>
<td>[132]</td>
</tr>
<tr>
<td>HDGF-B-Strep</td>
<td>Human HDGF-B sequence was cloned into BamHI / EcoRI pcDNA3Amp Strep-tag vector</td>
<td>[132]</td>
</tr>
<tr>
<td>HDGF-B_CEYFP</td>
<td>CEYFP (aa 173-238 of EYFP) fragment was cloned using EcoRI and XhoI restriction sites of pcDNA3Amp followed by in frame cloning of the HDGF-B using HindIII and BamHI restriction</td>
<td></td>
</tr>
<tr>
<td>HDGF-B_NFYFP</td>
<td>NEYFP (aa 1-172 of EYFP) fragment was cloned using EcoRI and XhoI restriction sites of pcDNA3Amp followed by in frame cloning of the HDGF-B using HindIII and BamHI restriction</td>
<td></td>
</tr>
<tr>
<td>HDGF-C (untagged)</td>
<td>Human HDGF-C sequence was cloned into EcoRI / BamHI digested pcDNA3Amp vector</td>
<td>[132]</td>
</tr>
<tr>
<td>HDGF-C-Strep</td>
<td>Human HDGF-C sequence was cloned into BamHI / EcoRI digested pcDNA3Amp Strep-tag vector</td>
<td>[132]</td>
</tr>
<tr>
<td>HDGF-C_CEYFP</td>
<td>CEYFP (aa 173-238 of EYFP) fragment was cloned using EcoRI and XhoI restriction sites of pcDNA3Amp followed by in frame cloning of the HDGF-C using HindIII and BamHI restriction</td>
<td></td>
</tr>
<tr>
<td>HDGF-C_NFYFP</td>
<td>NEYFP (aa 1-172 of EYFP) fragment was cloned using EcoRI and XhoI restriction sites of pcDNA3Amp followed by in frame cloning of the HDGF-C using HindIII and BamHI restriction</td>
<td></td>
</tr>
<tr>
<td>Human HDGF-A S165A (untagged)</td>
<td>Human HDGF S165A was generated by site-directed mutagenesis and sequence was cloned into EcoRI / BamHI digested pcDNA3Amp vector</td>
<td>Frank Dietz</td>
</tr>
<tr>
<td>Lamin B1-Strep</td>
<td>Lamin B1 was amplified as described in [49] and sequence was cloned N-terminal in frame to the Strep tag into the digested BamHI/EcoRI pcDNA3Amp Strep-tag vector</td>
<td>Frank Dietz</td>
</tr>
<tr>
<td>Luciferase-Strep</td>
<td>pMCS-Gaussia Luc vector (Thermo Scientific, Schwabach, Germany) was used as a template to amplify the Gaussia luciferase (GLuc) GLuc sequence was cloned into BamHI/EcoRI digested pcDNA3Amp Strep vector</td>
<td>Frank Dietz</td>
</tr>
<tr>
<td>Murine HDGF wt (untagged)</td>
<td>Murine HDGF sequence was cloned into HindIII / BamHI digested pcDNA3Amp vector</td>
<td>[33]</td>
</tr>
<tr>
<td>Murine HDGF C12A (untagged)</td>
<td>HDGF mutants were generated by site-directed mutagenesis and cloned into pcDNA3Amp vector</td>
<td>[33]</td>
</tr>
<tr>
<td>Murine HDGF C108A (untagged)</td>
<td>Mutant of murine HDGF was generated by site directed mutagenesis and cloned into pcDNA3Amp vector</td>
<td>[33]</td>
</tr>
<tr>
<td>Murine HDGF C12/108A (untagged)</td>
<td>Mutant of murine HDGF was generated by site directed mutagenesis and cloned into pcDNA3Amp vector</td>
<td>[33]</td>
</tr>
<tr>
<td>Murine HDGF S102/103A (untagged)</td>
<td>Mutant of murine HDGF was generated by site directed mutagenesis and cloned into pcDNA3Amp vector</td>
<td>[33]</td>
</tr>
</tbody>
</table>
### Material and Methods

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine HDGF S132A (untagged)</td>
<td>Mutant of murine HDGF was generated by site directed mutagenesis and cloned into pcDNA3 Amp vector</td>
</tr>
<tr>
<td>Murine HDGF S133A (untagged)</td>
<td>Mutant of murine HDGF was generated by site directed mutagenesis and cloned into pcDNA3 Amp vector</td>
</tr>
<tr>
<td>Murine HDGF S132/S133A (untagged)</td>
<td>S132A was used as template for preparation of the double mutants by performing site directed mutagenesis. Sequence was cloned into EcoRI / BamHI digested pcDNA3 Amp vector</td>
</tr>
<tr>
<td>Murine HDGF S165A (untagged)</td>
<td>Mutant of murine HDGF was generated by site directed mutagenesis and cloned into pcDNA3 Amp vector. Sequence was cloned into EcoRI / BamHI digested pcDNA3 Amp vector</td>
</tr>
<tr>
<td>Murine HDGF S132/S165A (untagged)</td>
<td>S132A was used as template for preparation of the double mutants by performing site directed mutagenesis as described by [33]. Sequence was cloned into EcoRI / BamHI digested pcDNA3 Amp vector</td>
</tr>
<tr>
<td>Murine HDGF S132/S133/S165A (untagged)</td>
<td>S132/133A mutant was the base for the triple mutant by performing site directed mutagenesis as described by [33]. Sequence was cloned into EcoRI / BamHI digested pcDNA3 Amp vector</td>
</tr>
<tr>
<td>Murine HDGF K8R Strep</td>
<td>Murine HDGF-Strep was used as template for the preparation of K8R by performing site directed mutagenesis as described by [33]. Sequence was cloned into EcoRI / BamHI digested pcDNA3 Amp vector</td>
</tr>
<tr>
<td>Murine HDGF K11R Strep</td>
<td>Murine HDGF-Strep was used as template for the preparation of K11R by performing site directed mutagenesis as described by [33]. Sequence was cloned into EcoRI / BamHI digested pcDNA3 Amp vector</td>
</tr>
<tr>
<td>NΔ8 HDGF (untagged)</td>
<td>Murine HDGF N-terminally truncated fragment, deleted in first 8 N terminal amino acids, was cloned into BamHI / EcoRI digested vector pcDNAIII Amp</td>
</tr>
<tr>
<td>NΔ10 HDGF (untagged)</td>
<td>Murine HDGF N-terminally truncated fragment, deleted in first 10 N terminal amino acids, was cloned into BamHI / EcoRI digested vector pcDNAIII Amp</td>
</tr>
<tr>
<td>NΔ11 HDGF (untagged)</td>
<td>Murine HDGF N-terminally truncated fragment, deleted in first 11 N terminal amino acids, was cloned into BamHI / EcoRI digested vector pcDNAIII Amp</td>
</tr>
<tr>
<td>NΔ12 HDGF (untagged)</td>
<td>Murine HDGF N-terminally truncated fragment, deleted in first 12 N terminal amino acids, was cloned into BamHI / EcoRI digested vector pcDNAIII Amp</td>
</tr>
<tr>
<td>NΔ15 HDGF-A (Strep-tagged)</td>
<td>Murine HDGF N-terminally truncated fragment, deleted in first 15 N terminal amino acids, was cloned N-terminal in frame to the Strep-tag into the BamHI / EcoRI digested pcDNA3 Strep</td>
</tr>
</tbody>
</table>
### Material and Methods

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N10 HDGF SNAP-Strep</td>
<td>N-terminal HDGF sequence, containing the first 10 aa of HDGF, was obtained as described by [33] and cloned N-terminal into the EcoRI/NotI digested pcDNA3 Amp vector. Simultaneously, the coding sequence of the hAGT protein (SNAP) was amplified using the pSNAP-tag (m) vector (NEB, Ipswich) as a template and subcloned in frame into BamHI/EcoRI digested pcDNA3 Strep vector. Vector was BamHI/NotI double digested to obtain SNAP-StrepTag sequence and this sequence was ligated to BamHI/NotI digested pcDNA3, containing the N-terminal peptide of HDGF.</td>
<td>[33]</td>
</tr>
<tr>
<td>N15 HDGF SNAP-Strep</td>
<td>N-terminal sequence, containing the first 15 aa, was amplified from HDGF and cloned into EcoRI/NotI digested pcDNA3 Amp vector. As described above, SNAP-Strep sequence was cloned in frame to the HDGF peptide using BamHI and NotI restriction site.</td>
<td>Frank Dietz</td>
</tr>
<tr>
<td>N30 HDGF SNAP-Strep</td>
<td>N-terminal sequence, containing the first 30 aa of HDGF, was amplified from HDGF and cloned into EcoRI/NotI digested pcDNA3 Amp vector. As described above, SNAP-Strep sequence was cloned in frame to the HDGF peptide using BamHI and NotI restriction site.</td>
<td>Frank Dietz</td>
</tr>
<tr>
<td>pcDNA3 Amp</td>
<td>Mammalian Expression Vector (vector map in the appendix)</td>
<td>Invitrogen, Carlsbad</td>
</tr>
<tr>
<td>pcDNA3 Strep</td>
<td>Mammalian Expression Vector</td>
<td>[49]</td>
</tr>
<tr>
<td>pEGFP N3</td>
<td>Mammalian Expression Vector (vector map in the appendix)</td>
<td>Clontech, Palo Alto, CA, USA</td>
</tr>
<tr>
<td>pEXPR IBA5</td>
<td>Mammalian Expression Vector (vector map in the appendix)</td>
<td>IBA, Göttingen</td>
</tr>
<tr>
<td>Snap-Strep</td>
<td>The coding sequence of the hAGT protein (SNAP) was amplified using the pSNAP-tag (m) vector (NEB, Ipswich) as a template and subcloned in frame into BamHI/EcoRI digested pcDNA3 Strep vector.</td>
<td>Frank Dietz</td>
</tr>
</tbody>
</table>
### 2.2.8 Antibodies

#### 2.2.8.1 Primary antibodies

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Company/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti pan-HDGF, polyclonal</td>
<td>1:2000 (Wb); 1:1000 (IF)</td>
<td>R&amp;D systems, Minneapolis</td>
</tr>
<tr>
<td>Goat anti-kinesin 1, polyclonal</td>
<td>1:500 (Wb); 1:200 (IF)</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Mouse anti-actin, pan 5, monoclonal</td>
<td>1:2000 (Wb); 1:1000 (IF)</td>
<td>Dianova GmbH, Hamburg</td>
</tr>
<tr>
<td>Mouse anti-dynein IC, 74.1 (74 kDa); monoclonal</td>
<td>1:250 (Wb); 1:200 (IF)</td>
<td>Thermo Fisher Scientific, Waltham</td>
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<tr>
<td>Mouse anti-HDGF (H3); monoclonal</td>
<td>1:500 (IF)</td>
<td>Santa Cruz, Dallas</td>
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<tr>
<td>Mouse anti-Hsc70 antibody 13D3; monoclonal</td>
<td>1:1000 (Wb)</td>
<td>Thermo Fisher Scientific, Waltham</td>
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<td>Mouse anti-nucleolin antibody ZN004; monoclonal</td>
<td>1:2000 (Wb); 1:1000 (IF)</td>
<td>Thermo Fisher Scientific, Waltham</td>
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<td>Mouse anti-tubulin DM1A, monoclonal</td>
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<td>Mouse anti-vimentin antibody; monoclonal</td>
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<tr>
<td>Rabbit anti HDGF-A, -B and -C (isoform specific peptide antibodies)</td>
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<tr>
<td>Rabbit anti human HDGF, polyclonal</td>
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<td>Frank Dietz</td>
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<tr>
<td>Sheep anti mouse HDGF; polyclonal</td>
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<td>Sheep anti-Strep Tag</td>
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#### 2.2.8.2 Secondary antibodies

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<tr>
<td>Cy3-conjugated donkey anti-rabbit antibody</td>
<td>1:500</td>
<td>Dianova, Hamburg</td>
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<tr>
<td>Cy5-conjugated donkey anti-mouse antibody</td>
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<td>Peroxidase-conjugated affinity purified donkey anti-goat IgG</td>
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<tr>
<td>Peroxidase-conjugated affinity purified donkey anti-mouse IgG</td>
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<td>Dianova, Hamburg</td>
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<tr>
<td>Peroxidase-conjugated affinity purified donkey anti-rabbit IgG (H+L)</td>
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<td>Dianova, Hamburg</td>
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<tr>
<td>Peroxidase-conjugated affinity purified donkey anti-sheep IgG (H+L)</td>
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### 2.2.9 Devices

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<td>ApoTome, Axio Imager Z1</td>
<td>Zeiss, Jena</td>
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### Devices and Company

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<th>Company</th>
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<tr>
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<td>Eppendorf AG, Hamburg</td>
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<tr>
<td>Centrifuge rotor (Sorvall evolution RC)</td>
<td>Sorvall GmbH, Bad Homburg</td>
</tr>
<tr>
<td>DelsaTM Nano C particle analyser</td>
<td>Beckman Coulter, Krefeld</td>
</tr>
<tr>
<td>Electrical power supply (SDS PAGE)</td>
<td>Biolog Laboratories GmbH, München</td>
</tr>
<tr>
<td>Electrical power supply (Western blotting)</td>
<td>Biolog Laboratories GmbH, München</td>
</tr>
<tr>
<td>Electrophoresis chamber</td>
<td>Biolog Laboratories GmbH, München</td>
</tr>
<tr>
<td>Fluorescence microscope</td>
<td>Olympus, Hamburg</td>
</tr>
<tr>
<td>Heating block</td>
<td>Eppendorf AG, Hamburg</td>
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<td>Ice machine</td>
<td>Scotsman Ice Systems, Frimont</td>
</tr>
<tr>
<td>Incubator (Bacteria) Unitron</td>
<td>Infors AG, Bottmingen</td>
</tr>
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<td>Incubator (Cell Culture)</td>
<td>Heraeus Holding GmbH, Hanau</td>
</tr>
<tr>
<td>Laminar air flow system</td>
<td>Heto Holten GmbH, Wettenberg</td>
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<tr>
<td>Magnetic stirrer</td>
<td>IKA Werke GmbH &amp; Co KG, Staufen</td>
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<tr>
<td>MALDI-TOF mass spectrometer</td>
<td>Bruker Daltonics GmbH, Bremen</td>
</tr>
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<td>MicroAmp Fast Optical 96-Well Reaction Plate</td>
<td>Thermo Fisher Scientific, Waltham</td>
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<td>Multipipette</td>
<td>Eppendorf AG, Hamburg</td>
</tr>
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<td>Nanodrop</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Neubauer counting chambers</td>
<td>Brandt GmbH &amp; Co. KG, Wertheim</td>
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<tr>
<td>pH Meter</td>
<td>Inolab WTW GmbH; Weilheim</td>
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<tr>
<td>Photometer</td>
<td>Eppendorf AG, Hamburg</td>
</tr>
<tr>
<td>Pipettes</td>
<td>Eppendorf AG, Hamburg</td>
</tr>
<tr>
<td>Pipette boy</td>
<td>Eppendorf AG, Hamburg</td>
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<tr>
<td>Refrigerator (4 °C)</td>
<td>Liebherr AG, Bremen</td>
</tr>
<tr>
<td>Refrigerator (-20 °C)</td>
<td>Vestfrost, Esbjerg</td>
</tr>
<tr>
<td>Refrigerator (-80 °C)</td>
<td>Eppendorf, AG Hamburg</td>
</tr>
<tr>
<td>Shaker Vibramax 100</td>
<td>Heidolph Instruments GmbH, Schwabach</td>
</tr>
<tr>
<td>StepOnePlus™ Real Time PCR Cycler</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Thermocycler</td>
<td>Eppendorf AG, Hamburg</td>
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<td>Ultracentrifuge, Le-80K</td>
<td>Beckman Coulter, Krefeld</td>
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<td>Ultracentrifuge, Optima MaxE</td>
<td>Beckman Coulter, Krefeld</td>
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<td>Ultracentrifuge rotor, Ti45</td>
<td>Beckman Coulter, Krefeld</td>
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<tr>
<td>Ultracentrifuge rotor, TLA 55</td>
<td>Beckman Coulter, Krefeld</td>
</tr>
<tr>
<td>Ultrasonication Sonifier B12</td>
<td>Banson Sonic Power Company, Danbury</td>
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<tr>
<td>Vacuum centrifuge</td>
<td>Thermo Fisher Scientific, Waltham</td>
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<td>Vortex</td>
<td>IKA Werke GmbH &amp; Co KG, Staufen</td>
</tr>
<tr>
<td>Vortex</td>
<td>IKA Werke GmbH &amp; Co KG, Staufen</td>
</tr>
<tr>
<td>Weighing Balance</td>
<td>Sartorius AG, Göttingen</td>
</tr>
<tr>
<td>Weighing Balance (fine measurements)</td>
<td>Sartorius AG, Göttingen</td>
</tr>
<tr>
<td>Western blotting device (tank)</td>
<td>Biolog Laboratories GmbH, München</td>
</tr>
<tr>
<td>X-ray film developing machine (Curix)</td>
<td>AGFA Gevaert, Mortsel</td>
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<tr>
<td>X-ray film cassette</td>
<td>GE Healthcare Europe GmbH, München</td>
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#### 2.2.10 Consumables

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<th>Consumables</th>
<th>Company</th>
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<tbody>
<tr>
<td>96 well microtitre plate</td>
<td>Greiner Bio-One GmbH, Frickenhausen</td>
</tr>
<tr>
<td>Amersham Hyperfilm ECL</td>
<td>GE Healthcare Europe GmbH, München</td>
</tr>
<tr>
<td>Blotting paper</td>
<td>GE Healthcare Europe GmbH, München</td>
</tr>
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</table>
Material and Methods

## Consumables

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell filter (70 µm)</td>
<td>Sarstedt AG &amp; Co. KG, Nümbrecht</td>
</tr>
<tr>
<td>Cell scraper</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>UltraClear UV, Richfield</td>
</tr>
<tr>
<td>Glass Cover Slips</td>
<td>Heinz Herenz Medizinalbedarf GmbH, Hamburg</td>
</tr>
<tr>
<td>Mini Protean Precast gels TGX</td>
<td>BioRad Laboratories GmbH, München</td>
</tr>
<tr>
<td>Reaction tubes (1.5 mL)</td>
<td>Eppendorf AG, Hamburg</td>
</tr>
<tr>
<td>Reaction tubes (15 and 50 mL)</td>
<td>Sarstedt AG &amp; Co. KG, Nümbrecht</td>
</tr>
<tr>
<td>Parafilm</td>
<td>American National Can, New Jersey</td>
</tr>
<tr>
<td>Pasteur pipettes (glas)</td>
<td>Brand GmbH &amp; Co. KG, Wertheim</td>
</tr>
<tr>
<td>Pipette tips</td>
<td>Sarstedt AG &amp; Co. KG, Nümbrecht</td>
</tr>
<tr>
<td>Photometer plastic cuvettes</td>
<td>Brand GmbH &amp; Co. KG, Wertheim</td>
</tr>
<tr>
<td>PVDF membrane Hybond P</td>
<td>Carl Roth GmbH &amp; Co. KG, Karlsruhe</td>
</tr>
<tr>
<td>Scalpel</td>
<td>Braun GmbH, Melsungen</td>
</tr>
<tr>
<td>Serological pipettes (5 mL, 10 mL, 25 mL)</td>
<td>Sarstedt AG &amp; Co. KG, Nümbrecht</td>
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<tr>
<td>StrepTactin sepharose</td>
<td>IBA GmbH, Göttingen</td>
</tr>
<tr>
<td>Tissue cell culture plates (10 cm)</td>
<td>Sarstedt AG &amp; Co. KG, Nümbrecht</td>
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<tr>
<td>Tissue cell culture plate (3.5 cm)</td>
<td>Sarstedt AG &amp; Co. KG, Nümbrecht</td>
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<tr>
<td>Tissue cell culture plates (6; 24 ; 96 well)</td>
<td>Sarstedt AG &amp; Co. KG, Nümbrecht</td>
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### 2.2.11 Protein and DNA ladder

**MassRuler DNA Ladder Mix**
Thermo Fisher Scientific, Waltham
Contains following fragment sizes (in bp): 80, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1031, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 10000

**Prestained Protein Ladder:**
Thermo Fisher Scientific, Waltham
Contains following fragment sizes (in kDa): 10, 15, 25, 35, 40, 55, 70, 100, 130, 170

### 2.2.12 Kits

<table>
<thead>
<tr>
<th>Kit</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>BCA Protein Assay Kit</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>Cell Proliferation ELISA, BrdU (chemiluminescent)</td>
<td>Roche GmbH, Basel</td>
</tr>
<tr>
<td>ECL advanced Western blotting detection kit</td>
<td>GE Healthcare Europe GmbH, München</td>
</tr>
<tr>
<td>Fast Digest restriction enzymes</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>GeneJET Gel Extraction Kit</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>GeneJET Plasmid Miniprep Ki</td>
<td>Thermo Fisher Scientific, Waltham</td>
</tr>
<tr>
<td>NucleoBondR Xtra Midi kit</td>
<td>Macherey Nagel, Düren</td>
</tr>
<tr>
<td>Pierce ECL Western blotting substrate</td>
<td>Pierce Biotechnology, Rockford</td>
</tr>
<tr>
<td>Revert Aid First Strand cDNA synthesis kit</td>
<td>Thermo Fisher Scientific, Waltham</td>
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3. Results

3.1 Identification and verification of alternatively spliced forms of HDGF

3.1.1 Database analysis: HDGF-A in comparison to HDGF-B and HDGF-C

Alternative splicing is a mechanism which results in high diversity of proteins. Studies estimated that 96% of human genes undergo alternative splicing [133, 134]. Screening the NCBI database (www.ncbi.nlm.nih.gov/entrez) demonstrated that human HDGF belongs to this group of genes. Apart from the basic HDGF form, now termed HDGF-A during the course of this thesis, the sequences of two further N-terminal spliced sequences were recorded in the database, termed now HDGF-B (transcript variant 1; accession number NM_001126050.1) and HDGF-C (transcript variant 2; accession number NM_001126051). These sequences were identified in different cDNA libraries obtained from several human tissues. Respective accession numbers, length of mRNA transcript and of the resulting protein, as well as their respective molecular weights are given in table 5.

<table>
<thead>
<tr>
<th>Name in this study</th>
<th>HDGF-A</th>
<th>HDGF-B</th>
<th>HDGF-C</th>
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<tr>
<td>Accession number nucleic acids</td>
<td>NM_004494.2</td>
<td>NM_001126050.1</td>
<td>NM_001126051</td>
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<tr>
<td>Transcript name NCBI database</td>
<td>transcript variant 1</td>
<td>transcript variant 2</td>
<td>transcript variant 3</td>
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<td>Species</td>
<td>Homo sapiens</td>
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<td>Homo sapiens</td>
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<tr>
<td>Length mRNA transcript</td>
<td>2397 bp</td>
<td>2208 bp</td>
<td>2183 bp</td>
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<tr>
<td>Length coding sequence</td>
<td>723 nt</td>
<td>771 nt</td>
<td>702 nt</td>
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<td>Accession number protein</td>
<td>NP_004485.1</td>
<td>NP_001119522.1</td>
<td>NP_00119523.1</td>
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<tr>
<td>Protein name NCBI database</td>
<td>Isoform A</td>
<td>Isoform B</td>
<td>Isoform C</td>
</tr>
<tr>
<td>Length protein (aa)</td>
<td>240 aa</td>
<td>256 aa</td>
<td>233 aa</td>
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<tr>
<td>Calculated molecular weight</td>
<td>26.8 kDa</td>
<td>28.3 kDa</td>
<td>25.5 kDa</td>
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</table>

In the human genome the HDGF gene is located on chromosome 1 (1q23.1) [135]. As shown in the splicing scheme (figure 6 A), the three HDGF isoforms are encoded by six exons. Isoform HDGF-B and isoform HDGF-C result from the usage of a first alternative
in-frame exon. In all three variants exon 1(a/b/c) - 3 are spliced to exon 5 - 7, leaving out exon 4.

**Figure 6: Alternative splicing of HDGF.** A) Intron-exon organisation of HDGF-A, HDGF-B and HDGF-C. Exons 1-7 are depicted as boxes. Isoforms HDGF-A, -B and -C are encoded by six exons. The first three exons (1-3) are spliced to exon 5, 6 and 7 as shown in the schematic depiction. The alternative isoforms result from usage of a first alternative exon (1A/1B/1C). (B) Alignment of all resulting HDGF variants. (C) Alignment of HDGF variants displaying the presence of motifs (NLS1 & NLS2) and domains (red box = PWWP domain).

The resulting novel isoforms HDGF-B and C differ slightly in their amino acid length and resulting from that, in their molecular weight (table 5).

Since alternative splicing based on the usage of a first alternative exon, the generated isoforms HDGF-A, -B and -C consist of a different N-terminal part, while the C-terminal part remains unchanged. An amino acid alignment of HDGF-B and HDGF-C to HDGF-A (figure 7) revealed the affected parts of the proteins.
Figure 7: Amino acid alignment of the novel HDGF isoforms HDGF-A, -B and -C. Amino acid sequences, given in single letter code, are aligned. Identical amino acids are on a dark gray background, amino acids identical only in two HDGF isoforms are on a light gray background. The PHWP motif (aa 24-27 in HDGF-A) is highlighted by a box. Above the amino acid sequence the secondary structure of HDGF-A is depicted. The HATH region consists of five β-sheets 1-5 (blue arrows) followed by two α-helices (1&2) (red rectangles). Green rectangles depict the flexible loops between β-sheets and α-helices. Identified residues in HDGF-A for posttranslational modifications are highlighted by coloured box (red: phosphorylation Y23 [136], S103, T200 [36], S107 [137], S132, S133, S165 [34], S199, S202, S206, S239 [35], T225 [138] green: ubiquitination K11 [139]; K226 [140] yellow: acetylation K44 [141]; pink: SUMOylation K80 [49]).

The alignment demonstrated that HDGF-B and HDGF-C lack those amino acid residues of HDGF-A that constitute the first and the majority of the second β-sheet in HDGF-A (depicted by a blue arrow 1 & 2 in figure 7). The PHWP motif, which is located in the second β-strand of HDGF-A (highlighted by a black box in figure 7), is replaced by Arg40-Ala41-Thr42-Pro43 at the corresponding positions in HDGF-B. Respectively, only the fourth position of this motif is maintained in HDGF-B. Pro43 of HDGF-B corresponds to the Pro27 in HDGF-A.

In HDGF-C no obvious similarities to the PHWP motif are available. Instead of the PHWP motif, Cys17-Ala18-Gly19-Ala20 can be found in HDGF-C at the corresponding position in the alignment.

HDGF-A is a phosphoprotein with several potential phosphorylation sites mainly in its C-terminal part (in the sequence highlighted by red boxes, figure 7) [34, 35, 136-138]. Moreover, an acetylation site (Lys44) (yellow box) [141], a SUMOylation site (Lys80) (pink box) [49] and two ubiquitylation sites (Lys11 and Lys226) (green boxes) [139, 140] have been reported in HDGF-A. Most of these sites were identified by large mass spectrometry proteome analysis and the function or biological relevance is barely known [34, 35, 136-141]. As shown in the alignment, these sites, with exception of Lys11 and Tyr23 are also potentially available in HDGF-B and HDGF-C.
Human HDGF-A and respective mouse HDGF share a high level of similarity (86 %). Furthermore, the number and the length of HDGF coding exons are the same in both species. However, alignments of the respective gene sequences demonstrated that the alternative first exons (1b and 1c), encoding the N-terminal part of HDGF-B and HDGF-C, were not present in the mouse genome. Performing blast search led to the discovery that apart from *Homo sapiens* both alternative isoforms have been also predicted for different Old World monkeys (*Cercopithecidae*) (HDGF-B: *Pan paniscus*, *Pongo abelii*, *Gorilla gorilla*, *Macaca fascicularis*, *Mandrillus leucophaeus*) (HDGF-C: *Pan paniscus*).

### 3.1.2 Gene expression of HDGF isoforms in human cell lines

To verify gene expression of reported HDGF isoforms -A, -B, and -C, RNA was isolated from different human cell line (HeLa, HepG2, MCF-7, MDA-MB-231) and transcribed into cDNA. Subsequent PCR was performed using specific primer against the mentioned HDGF isoforms. In order to detect the obtained amplification products, agarose gelelectrophoresis was performed. The respective record of the gel is shown in figure 8.

![Gene expression of HDGF isoforms in different human cell lines.](image)

Amplification products contained an expected size of 727 bp (HDGF-A), 783 bp (HDGF-B) and 714 bp (HDGF-C). Respective bands were observed at the expected position in the agarose gel (figure 8). Therefore, all isoforms were found to be expressed as mRNA in the studied cell types.

As next step, sequences were cloned into pcDNA3 Amp vector (*BamHI, EcoRI* restriction sites) and resulting constructs were sequenced in order to validate the obtained PCR fragments. In all cases, the determined sequences matched with the reported sequences found in the NCBI database (data not shown) (corresponding NCBI accession numbers for each sequence of the HDGF isoforms are given in table 5).
3.1.3 Quantification of HDGF isoform expression in different human cell lines

The analysis of expression pattern of several alternative isoforms within different cell lines demonstrated that several isoforms, deriving from the same gene, are often simultaneously expressed, but that in general one isoform is the dominant expressed isoform [142].

In order to analyse the expression pattern of the novel HDGF isoforms HDGF-B and -C relative to HDGF-A and, furthermore, also to explore which of these isoforms is the main expressed form, real-time qPCR analysis was performed as described under section 2.1.1.5. Therefore, cDNA was used from different human cell types (MDA-MB-231, MCF-7, human dermal fibroblasts, HeLa, and HepG2). The obtained results are shown in figure 9 & 10.
Figure 9: Comparison of relative mRNA levels of HDGF-A, HDGF-B and HDGF-C. Relative mRNA expression of the three transcripts of the HDGF gene (HDGF-A, -B, -C) was determined by real-time quantitative PCR in primary human dermal fibroblasts or in human cell lines HeLa, MCF-7, MDA-MB-231, HepG2. Each PCR was performed in triplicates and means were determined. Results were normalised to microglobulin expression. Vertical bars indicate expression of HDGF isoforms B and C relative to HDGF-A, which was set to 1. The standard deviation of triplicates is represented by error bars.

In all investigated cell lines all three isoforms of HDGF were expressed at the same time. This was in agreement with the results of the first RT-PCR shown in figure 8.
HDGF-A was clearly the dominant expressed form in all investigated cell types, whereas HDGF-B and HDGF-C were only expressed at very low levels in relation to HDGF-A. In breast cancer cell lines (MCF-7 and MDA-MB-231) HDGF-B and HDGF-C expression were similar while in dermal fibroblasts HDGF-C expression was slightly stronger than HDGF-B.

Comparing the expression of one HDGF isoform within one cell line exhibited distinct expression pattern for each isoform as shown in figure 10.

Noticeably, expression of all three isoforms in primary dermal skin fibroblasts from human skin was relatively small compared to the expression of the other immortalised cell lines.
3.1.4 Ectopic expression of HDGF-Isoform

3.1.4.1 Western blot analysis of ectopically expressed HDGF-Isoforms

In order to receive more information about the HDGF isoforms, the coding sequences were subcloned into the mammalian expression vector pcDNA3 Amp vector (BamHI, EcoRI restriction sites) or cloned in frame to the Strep-tag into pcDNA3 Amp Strep vector (BamHI, EcoRI restriction sites). Obtained constructs were transiently overexpressed in HepG2 cells. After an incubation time of 24 h cells were lysed and cleared lysates were subjected to Western blot analysis using an anti pan-HDGF antibody, which was able to detect all three variants (figure 11).

Figure 11: Ectopic expression of HDGF-A, HDGF-B and HDGF-C. HepG2 cells were transiently transfected with plasmids coding for untagged HDGF-A, HDGF-B and HDGF-C (panel 1) or with plasmids coding for C-terminal Strep-tagged HDGF isoforms (panel 2). Cells were lysed and respective cleared cell lysate with DTT (panel 1a, 2a & 2b) or without DTT (panel 1b) were separated on 12% SDS-PAGE gels, transferred to PVDF membranes and probed with goat anti pan-HDGF (panel 1a, 1b, 2b) or anti Strep-tag (panel 2a). Molecular mass marker lanes are shown on the left.
Human HDGF-A has a molecular weight of approximately 27 kDa (table 5), but in Western blot analysis it is usually detected at around 37 kDa (figure 11, panel 1a). Reason for this is an abnormal running behaviour during SDS PAGE [3]. Due to the presence of the Strep-tag, C-terminally Strep-tagged HDGF-A was detected at a slightly higher molecular weight position of 40 kDa compared to the untagged form. Also HDGF-B and HDGF-C migrated more slowly during SDS PAGE than was expected on the basis of their predicted molecular weights. The anti pan-HDGF antibody detected a triple band signal at positions of 37-40 kDa in those samples containing ectopically overexpressed untagged HDGF-B (figure 11, panel 1a). In contrast to this, the predicted molecular weight of HDGF-B was 28.3 kDa (table 5). The triple signal could be also observed for C-terminally Strep-tagged HDGF-B after detection with an anti pan-HDGF antibody at a slight higher position of approximately 39-44 kDa (panel 2b). The Strep-tag antibody only recognized the upper and lower signal, not the intermediate signal (panel 2a).

Ectopically expressed HDGF-C was observed at a molecular mass position of approximately 37 kDa (with Strep-tag at 40 kDa) (panel 1a, 2a respectively). By contrast, the predicted molecular weight of HDGF-C was only 25.5 kDa (table 5).

In summary, all three HDGF isoforms exhibited a quite comparable discrepancy between calculated molecular weight and observed molecular mass position in SDS PAGE gels. A further interesting observation was obtained when comparing the signals of samples treated with or without reducing agent DTT. Under non-reducing conditions (figure 11, panel 1b) an additional strong signal was detected at a molecular mass position of approximately 90 kDa mainly for HDGF-A. Such a signal was detected also for HDGF-B and HDGF-C, but to a much weaker extent. These HDGF polypeptides were susceptible to DTT, since the respective signal disappeared in the presence of DTT (panel 1a).

### 3.1.4.2 Subcellular localisation of ectopically expressed HDGF isoforms

In general HDGF-A is targeted to the nucleus of cells due to the presence of two NLS sequences [8]. In this context Everett et al. demonstrated in vascular smooth muscular cells that nuclear location is required for the mitogenic activity of ectopically expressed HDGF [28]. However, some studies revealed that HDGF can be also present in the cytoplasm of cells [1, 29, 37] indicating that nuclear localisation is not exclusive intercellular localisation.

In human HDGF-A NLS 1 encompasses aa 75 - 80, while the bipartite NLS 2 is positioned at aa 155 - 170 [8, 17]. As shown in the alignment (figure 7), the changes due to alternative splicing in N-terminal part of HDGF-B and HDGF-C did not affect these
Results

parts of the protein. This led to the expectation that all variants are mainly located in the nucleus.

To clarify whether both isoforms have the same location as HDGF-A in cells, Cos7 cells grown on glass cover slips, were transiently transfected with an expression vector encoding C-terminally Strep-tagged HDGF isoforms HDGF-A, -B and -C. After 24 h incubation, cells were fixed, permeabilized and stained with monoclonal pan-HDGF antibodies (H3) as well as Strep-tag antibodies. Immunostained cells were imaged using a Zeiss Apotome fluorescence imager. The image is shown in figure 12.

Figure 12: Subcellular localization of HDGF-A, HDGF-B and HDGF-C. Cos-7 cells were transfected with plasmids coding for C-terminal Strep-tagged HDGF isoforms as indicated on the left side (HDGF-A, HDGF-B and HDGF-C). After 24 h cells were fixed and permeabilized as described under section 2.1.4. Cells were co-stained using an anti Strep-tag (red) and monoclonal anti HDGF (H3) (green) antibody. Immunostaining was analysed by fluorescence microscopy. The right panel (merge) shows the superposition of the red and green signals. DAPI (blue) was used for nuclear staining. Scale bar = 10µM (right corner).

The application of Strep-tag antibodies (red signal), which recognized the C-terminal Strep-tag of the recombinant proteins, allowed the discrimination of ectopically expressed HDGF isoforms from the background caused by the presence of endogenous HDGF. Monoclonal anti-human HDGF (green signal) antibodies recognized both endogenous and recombinant protein. The majority of transfected Cos-7 showed a strong and dominant red fluorescence signal mainly in their nuclei. Therefore, besides HDGF-A also HDGF-B and HDGF-C were primarily translocated to the nucleus of cells.
Interestingly, compared to HDGF-A, HDGF-B and HDGF-C were more prominently detected in the cytoplasm of cells.

3.1.5 Isoform specific antibodies

Alternative splicing can produce a large variety of mRNA transcripts from the same gene [142]. However, not all alternatively spliced transcripts will result in expressed functional proteins [143]. In order to maintain high fidelity of gene expression different mechanisms exist on posttranscriptional level, which control the stability and decay of mRNAs and by that way influence subsequent translation into proteins [144]. Hence, the verification of the expressed protein is necessary.

The similar running behaviour in SDS PAGE (figure 11) complicated the discrimination and identification of HDGF isoforms. For this reason isoform specific antibodies were required in order to verify protein expression of HDGF-B and HDGF-C.

Most commercially available HDGF antibodies are raised against the C-terminal part of HDGF, which is unique part of HDGF within the HRP-family (see also figure 1). Consequently, these antibodies would allow discrimination between the different HRP family members but they cannot distinguish HDGF-A from the two novel HDGF isoforms HDGF-B and HDGF-C.

3.1.5.1 Design and characterisation of isoform specific antibodies

To develop isoform specific antibodies, the unique N-terminal peptides of each isoform were chosen as shown in table 6.

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDGF-A (1)</td>
<td>SRSNRQKEYKC_{12}</td>
</tr>
<tr>
<td>HDGF-A (2)</td>
<td>CGDLVFAKMGYPH_{25}</td>
</tr>
<tr>
<td>HDGF-B</td>
<td>CTKLKRFLS Kag GARRAQ_{34}</td>
</tr>
<tr>
<td>HDGF-C</td>
<td>EQRAGGNRQVTSTLN_{17}</td>
</tr>
</tbody>
</table>

Blast search demonstrated that selected peptide sequences of HDGF-A (1), -B and -C are indeed unique and there are not any other proteins at least in the NCBI database available with a similar peptide sequence. Interestingly, Nakamura et al. used initially an HDGF antibody, directed against the same polypeptide like selected for HDGF-A antibody (1) [1]. Since obtained HDGF-A antibody (1) did not showed any binding in
Results

Western blot analysis (data not shown), a second peptide sequence was selected as target sequence located more downstream from the first selected target sequence (table 6; HDGF-A (2)). However, this sequence overlapped with the N-terminal part of all other HRP-family, which share a high homology in the *hath* region [10] (figure 1). Therefore, this antibody is theoretically able to discriminate HDGF-A from the isoforms B and C, but will additionally recognize LEDGF, HRP-2, HRP-3 and HRP-4 due to the similarity in the *hath* region. To circumvent this problem, co-staining with an HDGF specific antibody would be necessary in this case.

The peptide production, immunisation and antibody purification was done by the company Biomatics (Kanada). Obtained affinity purified antibodies were tested for functionality and specificity. Therefore, the C-terminal *Strep*-tagged HDGF-isoforms HDGF-A, -B and -C were ectopically expressed in HepG2 cells and respective cleared cell lysates were subjected to Western blot analysis. The PVDF membranes were probed with the novel isoform specific antibodies and to exclude unspecific binding the pre-immune sera were tested as well. As mentioned, the peptide antibody HDGF-A (1) (table 6) was not functional and therefore was excluded from the subsequent studies. Instead the alternative peptide antibody (2) was used. The results of Western blot analysis are shown in figure 13.
Figure 13: Antibody characterisation of novel peptide antibodies. HepG2 cells were transiently transfected with mammalian expression plasmids coding for C-terminally Strep-tagged HDGF-A, HDGF-B and HDGF-C. Cells were lysed and respective cleared cell lysates were separated on 12 % SDS-PAGE gels, transferred to PVDF membranes and probed with novel peptide antibodies anti HDGF-A (2) (panel 1a), anti HDGF-B (panel 2a) or anti HDGF-C (panel 3a) or respective pre-immune sera (panel 1b, 2b, 3b). Same lysates were probed anti Strep-tag antibody (panel II) to verify the expression of the different HDGF isoforms. Marker lanes are shown on the left.

Only the application of anti HDGF-A (figure 13, panel 1a) and anti HDGF-C (figure 13, panel 3a) isoform specific antibodies gave a signal at expected position of approximately 40 kDa for the Strep-tagged isoforms. Both antibodies were specific, since only the
corresponding isoform was recognized and not the remaining two alternative splice variants. Detection with respective pre-immune sera (figure 13, panel 1b & 3b) caused just weak background, a signal at the identical position of the corresponding HDGF isoforms was not observed. The affinity purified isoform specific HDGF-A and HDGF-C antibodies did not show any unspecific signals at a different molecular weight position. Isoform specific HDGF-B antibody did not recognize the overexpressed HDGF-B variant (figure 13, panel 2a), although its transient expression was verified by the use of anti Strep-tag (figure 13, panel II). Furthermore, not only the application of the affinity purified antibody but also the application of the pre-immune sera resulted in signals at approximately 20 kDa.

Since lysates from HDGF expressing cells were used, there is of course endogenously expressed HDGF protein present in these samples. Hence, in all samples such a “background” staining in all three lanes should be visible. However, this was not the case, also after longer exposure times (data not shown).

There are several potential reasons why HDGF-B antibody did not work. The simplest explanation would be that the HDGF-B peptide antibodies were not functional and could not bind the epitope. A further explanation could be that the selected region, which is recognized by the antibody, might undergo posttranslational modifications, which interfere with antibody binding. It is also possible that the isoform HDGF-B was N-terminally truncated and therefore lost the epitope for antibody recognition. This would be also in line with the observation that transiently overexpressed HDGF produced triple signals in Western blot analysis (figure 11), which indicated the presence of several peptides of different length.

In order to find out, whether the last hypothesis is applicable, N-terminal Strep-tagged EGFP-HDGF isoform (-A/-B/-C) constructs were cloned as follows: EGFP coding sequence was amplified and cloned in frame to the Strep-tag into EcoRI/BamHI digested pEXPR vector. HDGF isoform specific sequences were inserted in frame to the Strep-tagged EGFP into BamHI and HindIII digested pEXPR EGFP vector. Obtained constructs were transiently expressed in HepG2 cells. Cleared lysates were subjected to Western Blot analysis. Results are shown in figure 14.
Results

Figure 14: N-terminally-tagged HDGF variants. HepG2 cells were transiently transfected with mammalian expression plasmids coding for N-terminally Strep-tagged EGFP-HDGF-A, HDGF-B and HDGF-C. Cells were lysed and respective cleared cell lysate were separated on 12 % SDS-PAGE gels, transferred to PVDF membranes and probed with novel peptide antibodies anti HDGF-A (2) (panel 1a), anti HDGF-B (panel 1b), anti HDGF-C (panel 1c) or anti Strep-tag (panel 2). Molecular mass marker lanes are shown on the left.

The resulting constructs were detected at a molecular weight position of approximately 70 kDa. Strep-tag antibody yielded in a signal for all three variants (figure 14, panel 2), proving their expression. The application of all three isoform specific antibodies led to the detection of a specific signal in the cell lysates containing the respective ectopically expressed N-terminally tagged isoform (figure 14, panel 1a-c). Several polypeptides were visible, indicating that degradation products were also present.

Strikingly, HDGF-B-antibody was able to recognize the N-terminal tagged form (figure 14, panel 1b) although it did not bind the N-terminally untagged HDGF-B variant in the previous experiment (figure 13, panel 2). Hence, the HDGF-B antibody is basically functional in Western blot analysis and able to bind the epitope. However, again an unspecific signal at approximately 20 kDa was visible after usage of HDGF-B specific antibody. This signal, which was present in all three cell lysate samples, was as prominent as the specific signal for the N-terminal EGFP tagged form.
3.1.5.2 Endogenous expression of HDGF isoforms

The characterisation of the peptide antibodies (figure 13 & 14) clearly demonstrated that in principle all three antibodies (in case of HDGF A the peptide antibody 2, table 6) are capable of binding the corresponding transiently overexpressed HDGF isoforms. In case of HDGF-B the N-terminal part of recombinant protein must be tagged to allow its detection (figure 14).

Moreover the isoform specific peptide antibodies were clearly able to distinguish between the three HDGF isoforms. In order to verify endogenous expression of the two novel isoforms HDGF-B and HDGF-C cell lysates from different cell lines (HaCat, MDA-MB-231, MCF-7, HepG2, HeLa) were collected, subjected to Western blot analysis and tested for the presence of respective endogenous expressed HDGF isoforms by using the novel isoform specific antibody.

Neither endogenously expressed HDGF-A nor HDGF-B and HDGF-C could be detected in any of the investigated cell lysates when probing the corresponding PVDF membranes with the three different peptide antibodies (data not shown). The results of the real-time qPCR analysis (figure 9) already indicated that HDGF-B and HDGF-C were only expressed at low levels in comparison to HDGF-A. Since a sufficient amount of protein must be available to allow detection in Western blot analysis, the low expression of HDGF-B and HDGF-C might be the reason why these two isoform could not be observed in the different cell lysates. However, in case of HDGF-A, endogenous expression should be sufficient to allow detection in Western blot analysis.

In a further approach immunoprecipitation was performed by using the isoform specific antibodies as described in the section 2.1.9.4. Immunoprecipitation allows crude isolation as well as enrichment of the target protein and thus facilitates detection of low concentrated proteins.

Confluent grown HepG2 cells were lysed, and obtained cell lysates were added to protein A beads followed by an incubation under rotary condition in order to pre-clear lysates. The pre-cleared cell lysates were mixed with respective isoform specific peptide antibodies (A, B, C) or with the anti pan-HDGF antibody (H in figure 15), which served as positive control. To precipitate the endogenously expressed protein, new protein A beads were added to the samples and samples were incubated over night. The obtained eluates, pre-clearing (precipitation without antibodies) as well as pan-HDGF antibody were loaded to 12 % SDS PAGE gel and were separated in SDS PAGE, transferred to PVDF membranes and probed with the respective antibodies. Results are shown in figure 15.
Figure 15: Immunoprecipitation of endogenously expressed HDGF-isoforms. As indicated above, immunoprecipitation was performed by using isoform specific peptide antibodies against HDGF-A (A), HDGF-B (B), HDGF-C (C) or goat anti pan-HDGF antibody (H) from HepG2 cell lysates. Immunoprecipitates (A, B, C, H), pre-clearing (PC) and 5 µg of pan-HDGF antibody (AB) were separated on 12 % SDS PAGE gels and electroblotted on PVDF membranes. HC = Heavy chain. Arrows point to HDGF isoforms. Membranes were probed with isoform specific antibodies against HDGF-A (1), HDGF-B (2), HDGF-C (3) and goat pan-HDGF antibody (4). Molecular mass marker lanes are shown on the left.

The strong signals at a molecular weight position of 55 kDa, visible in all eluate fractions (A, B, C, H) and in the antibody sample (AB) (figure 15, panel 1-4), resulted from the presence of the antibody's heavy chain (position is marked with HC), which was still present in the final eluate fractions.
Results

In the eluates from immunoprecipitation with the isoform HDGF-C specific peptide antibody a signal at a molecular weight position of 37 kDa was obtained after immunodetection with isoform HDGF-C specific antibody (figure 15, panel 3) as well as with the anti pan-HDGF antibody (figure 15, panel 4). This position corresponded to that of untagged recombinant HDGF-C as shown in figure 11. Hence, the HDGF-C antibody was able to precipitate a protein with the expected size in SDS PAGE and, moreover, the antibody was able to detect this protein in the subsequent Western blot analysis (panel 3), strongly suggesting that the antibody is able to bind directly to the respective protein. Since the anti pan-HDGF antibody was able to detect the same signal, the 37 kDa signal in the HDGF-C immunoprecipitate was indeed HDGF protein (panel 4). In the same immunoprecipitate two additional signals were present after detection with HDGF-C antibody at a position of 60 kDa and 70 kDa. Signals were not detected by the anti pan-HDGF antibody. Therefore, these signals derived from a source which did not contain HDGF proteins.

Conversely, immunoprecipitate obtained with anti pan-HDGF antibody, contained a protein at a position of 37 kDa in Western blot analysis, which could be detected by both antibodies (anti HDGF-C and anti pan-HDGF) accordingly (panel 3 & 4, IP H) (marked with an arrow). As shown before (figure 11), anti pan-HDGF recognized all three HDGF isoforms, thus, corresponding immunoprecipitates would contain all three isoforms on condition that all three isoforms were available in the cleared cell lysates.

The characterisation of isoform specific peptide antibodies clearly showed that HDGF-C antibody discriminated between the different HDGF isoforms and was only able to react with ectopically expressed HDGF-C (figure 13). Due to this, the signal in goat anti pan-HDGF immunoprecipitate detected by anti-HDGF-C clearly verified the presence of endogenously expressed HDGF-C.

Of note, the pre-clearing (PC), which was collected before the antibody was added to the samples, did not exhibit any signals. Consequently, there was not any unspecific binding to the protein A beads.

With regard to the isoform specific peptide antibody HDGF-A (2), the respective immunoprecipitate exhibited a weak signal at 55 kDa after detection with anti-HDGF-A (figure 15, panel 1, IP A). On the other side, the anti pan-HDGF antibody was also able to detect the signal in the immunoprecipitate of HDGF-A (panel 4). However, in the immunoprecipitates of goat anti pan-HDGF antibody such a 55 kDa signal could not be detected by the HDGF-A specific antibody (panel 1) as well as by the anti pan-HDGF antibody (panel 4). Furthermore, the size of 55 kDa did not correlate with the expected size of 37 kDa. Therefore, the antibody failed to precipitate and detect HDGF-A.
By contrast, the immunoprecipitation with HDGF-B yielded in several proteins, which were detected in the range of 70-100 kDa (panel 2) after probing the respective PVDF membrane with anti-HDGF-B. These signals could not be detected after using goat anti pan-HDGF antibody (panel 4) or were present in the corresponding immunoprecipitate (panel 2 & 4). Signals of the expected size of 38-40 kDa were not visible.

In summary, endogenous HDGF-C expression could be verified. However, the other two antibodies, directed against HDGF-A and HDGF-B, were not able to precipitate the respective HDGF isoform. Instead they appeared to detect proteins of different sizes. Therefore, further characterisation of these antibodies is necessary to clarify the identity of the respective proteins.

### 3.1.5.3 HDGF-C in human tissue extracts

In the investigated human cell lines HDGF-C expression was low and detection was only possible when proteins were enriched by immunoprecipitation. To find a condition in which HDGF-C is upregulated, would help to understand the biological role of this isoform and support the relevance of this isoform.

Because in the investigated human cell lines expressed HDGF-C only at very low levels, the primary aim was to see whether any condition is available which leads to detectable HDGF-C protein levels. Different human tissue extracts, kindly provided by Dr. Mirastchijski, were examined for the presence of HDGF-C. Since the different samples derived from different patients with different backgrounds, there is not any statistical relevance regarding the type of skin or tissue and which kind of illness it underwent. The samples were analysed by Western blot and the obtained result is shown in figure 16.
Results

Figure 16: HDGF-C expression in human tissues. Tissue extracts were lysed in TNE buffer, homogenized and cleared by centrifugation at 12 000 x g. Same amounts of different human tissue extracts, obtained from different patients, were analysed by Western blot using isoform specific anti HDGF-C (panel 1), goat anti pan-HDGF (panel 2), anti-actin (panel 3). Tissue extracts (1-6) are described in the caption on the right site. Molecular mass marker lanes are shown on the left.

In these samples the pan-HDGF antibody detected a main signal at a position of 37 kDa (figure 16, panel 1). Interestingly, in one sample (sample no 3) an additional double signal appeared at 40 kDa on the same membrane. Moreover, this double signal at 40 kDa was also recognized by the isoform C specific antibody, whereas this antibody did not recognize the 37 kDa signal (figure 16, panel 2). Therefore, it is likely that that the detected polypeptide in sample number 3 was HDGF-C. However, the position of 40 kDa in the gel was slightly higher compared to the 37 kDa signals obtained from overexpression (figure 11) and immunoprecipitations (figure 15).

3.2 Functional analysis and characterisation of HDGF isoforms

Alternative splicing is a way to modulate properties and functions of proteins [145]. This includes features such as altered protein binding properties [146, 147], intracellular localisation [148, 149], protein stability [150], or enzyme activities [151]. HDGF-B and HDGF-C might have complete different functions and biological roles compared to HDGF-A. To get a first impression about their functional roles, HDGF-B and HDGF-C were examined with regard to some of the known features of HDGF-A.
3.2.1 Transient overexpression of HDGF-A and -B promotes cell growth

Numerous studies demonstrated that HDGF-A acts as mitogen and enhances proliferation of a wide variety of cells [1, 2, 17, 28, 50, 152]. It is suggested that this functional property of HDGF-A is associated with cancer development and progression [45, 152, 153]. Against this background it is important to investigate whether the novel isoforms HDGF-B and HDGF-C possess same abilities. Cos7 cells were transiently transfected with HDGF isoforms HDGF-A, HDGF-B, HDGF-C and with pcDNA3 as mock condition. To determine the effect of HDGF overexpression on cellular proliferation, BrdU proliferation assay was performed as described under method section (2.1.5.1). In this assay BrdU, a synthetic nucleotide analogon, was used. When provided, cells can incorporate BrdU instead of the cellular nucleotide thymidine during DNA synthesis. Antibodies specific for BrdU can detect the incorporated BrdU and thereby enable measurement of proliferation [154].

The results of the BrdU proliferation assay are shown in figure 17.

**Figure 17: Overexpression of HDGF-A and -B enhances cell proliferation.** Cos7 cells were transiently expressed with vectors encoding HDGF isoforms HDGF-A, HDGF-B, HDGF-C (A, B, C) and empty vector (Mock). 24 h posttransfection under serum-free conditions cells were provided with nucleotide analogon BrdU for 6 h. As described under methods, cells were fixed, stained with anti-BrdU-POD, and incubated in substrate solution. Absorbance was measured at 450 nm. Experiment was performed three times with each experiment consisting of 10 replicates. Data are given as the mean ± standard deviation (n=30). Analysis of significance of differences was performed by ANOVA, followed by the Bonferroni post hoc test ***P ≤ 0.005, compared to mock.
As shown already in other studies for other cell types [2, 17], HDGF-A overexpression yielded in an increase of Cos7 cell proliferation compared to the mock transfection (empty vector) (P≤0.005). Likewise, HDGF-B transient overexpression had a similar enhancing effect on Cos7 cell proliferation compared to the mock transfection. However, the overexpression of HDGF-C did increased cell proliferation, suggesting that unlike HDGF-A and HDGF-B this isoform was not able to promote cell synthesis.

### 3.2.2 Chromatin association: HDGF-A and HDGF-C associate with chromatin

Chromatin is a macromolecule complex, which consists of nucleic acids and variety of different proteins [155]. Majority of PWWP domain-containing proteins have the ability to bind to this complex structure [20] and thereby influence features or processes such as transcription activity [25], DNA architecture [156] or DNA methylation [157, 158]. Previous studies provided evidence that HDGF bears the potential to bind DNA [16, 25]. This binding property is mediated by the PWWP domain of HDGF [16, 25, 159]. Furthermore, the binding property appeared to be sequence specific since Yang et al. were able to identify a 39 bp DNA fragment as a specific target sequence for HDGF [25].

As shown in the alignment (figure 7), HDGF-B and HDGF-C undergo remarkable changes in their N-terminal part, including the loss of the PHWP motif. In order to investigate whether these changes affect chromatin association, the chromatin-binding assay, adapted from Llano et al. [160] and Thakar et al. [49] with minor modifications, was performed (see section 2.1.5.2).

This assay exploits the property that chromatin-bound proteins are not released after cell lysis with non-ionic detergents such as Triton X-100 [160, 161]. For this purpose a combined treatment with DNase and salt is necessary [160, 162]. A schematic depiction of the assay, including the collected fractions S1, S2, T, P1, P2, is shown in figure 18, panel I. The results of the chromatin-binding assay for the HDGF isoforms are presented in figure 18, panel II.

Endogenous tubulin (panel II, 1b, 2b, 3b, 4b) served as internal control and its missing in the Triton-insoluble, nuclear fractions (P1, S2, P2) illustrated that there were no impurities caused by the S1 fraction containing Triton-soluble proteins (such as cytosolic proteins). Furthermore, to confirm assay specificity, ectopically overexpressed LaminB1-Strep was utilized as second control. The respective Western blot (panel II, 5a) showed strong signals in the P1 and P2 fractions and a weak signal in the T fraction. This was in line with the expectations, since other studies demonstrated already that LaminB1 is a matrix bound protein [163] and therefore enriched in the nuclear insoluble, high salt resistant fraction [164].
Results

I. Chromatin-binding assay of HDGF isoforms.

Panel I: Schematic depiction of the chromatin-binding assay which is showing the collected fractions: T (total lysate), S1 (Triton-soluble fraction), P1 (Triton-insoluble fraction: chromatin-bound, nuclear matrix-bound, insoluble proteins), S2 (chromatin-bound, DNase and high salt released fraction: chromatin-bound proteins), P2 (Triton-insoluble, DNase and high salt non-released fraction: insoluble, cytoskeletal, nuclear matrix protein). Panel II: HepG2 cells were transiently transfected with plasmids coding for HDGF isoforms HDGF-A-Strep (panel 1), HDGF-B-Strep (panel 2) and HDGF-C-Strep (panel 3) as well as for Snap-Strep (panel 4) and Lamin B1-Strep (panel 5). 24 h after transfection cells were lysed and the fractions were prepared as described under method section 2.1.5.2: 10 μg of each sample were loaded to 12 % SDS PAGE gel, separated and transferred to PVDF membranes. Membranes were probed with goat anti pan-HDGF (panel 1a, 2a, 3a), anti Strep-tag (panel 4a, 5a) or anti-tubulin (1b, 2b, 3b, 4b, 5b). Molecular mass marker lanes are shown on the left.

Figure 18: Chromatin-binding assay of HDGF isoforms.
For HDGF-A the same signal distribution was obtained as shown already by Thakar et al. [49, 165]. HDGF-A was present in the Triton-soluble (S1) and Triton-insoluble fractions (P1, P2, S2) (figure 18, panel II, 1a). Treatment with DNase and high salt caused the release of majority of HDGF-A, thus it was detected in the S1 fraction. A smaller amount remained in the P2 fraction, which consisted of insoluble proteins such as matrix and cytoskeleton proteins.

HDGF-C exhibited the same distribution (figure 18, panel II, 3a) as HDGF-A. Therefore, this protein was still able to bind to chromatin. Surprisingly, HDGF-B was found only in T and S1 fraction and was completely excluded from the Triton-insoluble fractions (P1, P2 and S2 fractions) (figure 18, panel II, 2a). Consequently, HDGF-B lost the ability to bind to chromatin and was additionally absent in the insoluble, DNase resistant, nuclear matrix fraction, although immunofluorescence analysis detected HDGF-B in the nucleus of Cos7 cells (figure 12). The analysis of Snap-Strep (figure 18, panel 4) protein demonstrated that the overexpression did not artificially lead to presence in the chromatin associated fractions.

### 3.2.3 Protein-protein interaction of HDGF isoforms

The analysis of the interactome of a protein can provide insight into molecular functions and might help to understand the biological role. As mentioned in the beginning HDGF-A is known to interact with a wide number of different proteins [5]. Moreover, it has the ability to form homodimers [26].

#### 3.2.3.1 Homo- and Heterodimerisation of HDGF isoforms

Many proteins are able to self-associate in order to form homodimers or even oligomers. Self-association is an important subgroup of protein-protein interactions and was proven for diverse functional proteins such as enzymes, transcription factors, or receptors. Accordingly, functional consequence of dimer or oligomer formation can be diverse [166]. HDGF belongs to this group of proteins. Sue et al. studied the underlying dimerisation mechanism and demonstrated that dimerisation depends on an unusual domain swap mechanism. This domain swap mechanism is mediated by the PWWP domain and results in a higher binding affinity to the glycosaminoglycan heparin [26]. Binding to heparin is known to be essential for the internalisation of HDGF and consequently cellular entry might be influenced [167].

In addition to this, Thakar et al. suggested the presence of disulfide bridged homodimers via Cys108 in mouse HDGF [33]. In the HRP-family dimerisation was also proven for LEDGF but whether PWWP domain of LEDGF is involved in the dimer formation such as
shown for HDGF was not investigated [168]. Furthermore, HDGF and HRP-2 are able to interact with each other and thereby forming heterodimers [165].

To study the possible formation of HDGF isoform specific homodimers or heterodimers, the bimolecular fluorescence complementation (BiFC) assay was used which allowed the investigation of protein-protein interactions in the living cell. The principle of the assay, depicted in figure 19 (panel I), based on the formation of a functional fluorescent EYFP protein complex when non-functional N-terminal and C-terminal fragments of EYFP are brought in close proximity. This occurs when two interaction partners, each fused to one part of EYFP, associate with each other [169].

**Figure 19: BiFC assay.** Panel I: Schematic diagram of the BiFC assay. The non-functional N-terminal and C-terminal fragment of EYFP are fused to the potential interaction partners A and B. Interaction of A and B enables the formation of a biomolecular fluorescent EYFP complex. Panel II: COS-7 cells were co-transfected with plasmids coding for the different EYFP fragment fusion constructs of HDGF-A, -B and -C or with the non-fused EYFP fragments as indicated at the sites. BiFC was performed for visualisation of HDGF isoform interactions. EYFP fragment complementation signals were detected 30 min after exposure to 30 °C and visualised by fluorescence microscopy. DAPI was used for nuclear staining. Scale bar = 10 µm (right corner).
Results

For this assay the three isoforms HDGF-A, -B and -C were fused to the N-terminal or C-terminal fragment of EYFP. The result of the BiFC assay is shown in figure 19, panel II. The BiFC assay revealed that all HDGF isoforms appeared to be able to form homodimers since respective combinations resulted in a positive nuclear EYFP signal. Furthermore, heterodimerisation between the different HDGF isoforms seemed to be possible as well because respective combinations showed also a positive EYFP signal. Interestingly, combinations with HDGF-A resulted in a more homogenously distributed nuclear signal compared to the HDGF-B/ HDGF-C combination where the respective signal appeared more condensed.

3.2.3.2 Interaction with other proteins

The analysis of potential interaction partners can provide insights into the biological role of the respective protein.

HDGF-A is able to interact with a wide variety of proteins suggesting that HDGF might have functions in different cellular processes. The multi-interactome study by Zhao et al. revealed the presence of at least 106 interactions partners [5]. Proteins participate in cellular processes such as RNA splicing, ribosome biogenesis, chromatin remodelling or DNA repair. Most interestingly, interaction depends mainly on the N-terminal hath region of HDGF. Since alternative splicing leads to the changes in the hath region, the question raised how these resulting changes in the hath region affect the interaction properties of the novel isoforms HDGF-B and HDGF-C.

3.2.3.2.1 Identification of putative interacting proteins of HDGF-B and HDGF-C in co-precipitates

To study whether the unique N-terminal parts of the isoforms have an impact on protein-protein interactions, the C-terminally Strep-tagged HDGF isoforms were transiently overexpressed in HepG2 cells. Strep-tagged proteins were precipitated from the cleared lysate using StrepTactin coated beads. Respective eluates were separated by SDS-PAGE and, in order to visualise potential co-precipitated interaction partners, gels were stained with Coomassie stain. The respective recording of the gel is shown in figure 20 (panel 1).
Figure 20: HDGF isoforms associate with different proteins. HepG2 cells were transiently transfected with a plasmid coding C-terminally Strep-tagged HDGF isoforms (-A, -B, -C) or empty vector (con). After 24 h incubation cells were lysed and HDGF-Strep variants were precipitated using StrepTactin beads. Precipitates and an aliquot of the respective cleared lysate (input) were separated by SDS-PAGE. Panel 1: MALDI-TOF MS analysis was performed from selected protein bands excised from the Coomassie stained SDS-PAGE gel and subjected to in-gel trypsin digestion. Identified co-precipitated proteins in SDS-PAGE gel are marked by numbered spots. Caption below lists the identified proteins with the respective number. 2) Subsequent to SDS PAGE, samples were transferred to PVDF membranes and PVDF membranes were immunodetected using indicated antibodies: anti-nucleolin (panel 2a), anti-YB-1 (panel 2b), anti-tubulin (panel 2c), anti-actin (panel 2d), anti-vimentin (panel 2e), anti-dynein (panel 2f), anti-kinesin antibody (panel 2g), anti-Hsc70 (panel 2h), respectively. To detect Strep-tagged HDGF isoforms anti Strep-tag antibody was used. Molecular mass marker lanes are indicated on the left.

The staining of the gels revealed a complex banding pattern, especially for HDGF-A and HDGF-C (figure 20, panel 1). In accordance to the multi-interactome study of Zhao et al.
Results

[5], the co-precipitates of HDGF-A but also of isoform HDGF-B and HDGF-C exhibited multiple bands, indicating the presence of various putative interaction partners. Some bands occurred in all three precipitates while others were only identified in one or two precipitates. Thus, interaction pattern varied from each other. This demonstrated that alteration in HDGF-B and HDGF-C influences at least partly the interactions to proteins.

To identify possible interaction partners, stained bands were excised from the gel and subjected to MALDI-TOF MS based peptide analysis (complete MALDI-TOF results can be found in the appendix, table A1). Respective excised bands are marked with a spot in figure 20, panel 1. Main information about the identified proteins is listed in table 7.

Table 7: Co-precipitated proteins identified by MALDI-TOF MS. Spot number and corresponding protein as well as Swiss Prot ID number, main function and molecular weight are listed.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Identified in co-precipitate of HDGF</th>
<th>Identified protein</th>
<th>Swiss Prot ID</th>
<th>Function</th>
<th>Molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A, B, C</td>
<td>heat shock cognate 71 kDa (Hsc70)</td>
<td>P11142</td>
<td>chaperone</td>
<td>70.9 kDa</td>
</tr>
<tr>
<td>2</td>
<td>B, C</td>
<td>tubulin alpha 1C</td>
<td>Q9BQE3</td>
<td>constituent of microtubules</td>
<td>49.9 kDa</td>
</tr>
<tr>
<td>2</td>
<td>B, C</td>
<td>tubulin alpha-8 chain</td>
<td>Q9NY65</td>
<td>constituent of microtubules</td>
<td>50.1 kDa</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>Y-box binding protein (YB-1)</td>
<td>P67809</td>
<td>DNA/RNA binding factor</td>
<td>35.9 kDa</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>actin, cytoplasmic 1</td>
<td>P60709</td>
<td>cytoskeletal protein</td>
<td>41.7 kDa</td>
</tr>
<tr>
<td>5</td>
<td>A, B, C</td>
<td>ADP/ATP Translocase 2</td>
<td>P05141</td>
<td>antiporter</td>
<td>32.9 kDa</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td>60S ribosomal protein L6</td>
<td>Q02878</td>
<td>ribosomal protein</td>
<td>33.4 kDa</td>
</tr>
</tbody>
</table>

All three isoforms co-precipitated heat shock cognate 70 (Hsc70) and ADP/ATP translocase 2. ADP/ATP translocase 2 has been already reported as interaction partner of HDGF-A by Zhao et al. [5].

In the co-precipitate of HDGF-B and -C a prominent band was visible at approximately 55 kDa. Subsequent MALDI-TOF-MS analysis revealed that these bands contained α- and β-tubulin. As a further cytoskeleton protein actin was found at around 40 kDa exclusively in the HDGF-C precipitate. Both cytoskeletal proteins have not been described as human HDGF interacting proteins before.
In addition to this, only HDGF-A co-precipitated the Ybox binding protein (YB-1), a protein which was described to be involved in a variety of DNA/RNA dependent processes [170]. Furthermore, the 60S ribosomal protein L6 was identified in the eluate of HDGF-A, which is a component of the 60S ribosomal subunit. Several other components of ribosomes were already discovered in the interactome of HDGF-A [5].

To affirm the identified interaction partners, HDGF co-precipitates were further analysed by Western blot using antibodies directed against tubulin, actin, Hsc70 and YB-1 (figure 14, panel 2).

In line with MALDI-TOF-MS results, Hsc70 could be detected in all three HDGF isoform precipitates (panel 2h). By contrast, YB-1 was only present in the precipitate of HDGF-A (panel 2b). Precipitates were examined also for the presence of nucleolin, an already known interaction partner of HDGF-A [5, 123]. Interestingly, HDGF-B and HDGF-C did not co-precipitate this nuclear protein.

Strikingly, α-tubulin could be detected in the precipitates of HDGF-B and HDGF-C whereas it was completely absent in the precipitate of HDGF-A (panel 2c). Furthermore, the microfilament protein actin was only precipitated by HDGF-C (panel 2d).

Due to the finding that tubulin and actin were able to interact with the novel HDGF isoforms, precipitates were analysed by Western blot for the presence of other proteins of the cytoskeleton. Vimentin as a representative of the intermediate filaments [171] as well as dynein and kinesin as tubulin-dependent motor proteins [172] were selected.

Also the intermediate filament vimentin could be detected only in the HDGF-C precipitate (panel 2e). Furthermore, HDGF-B and HDGF-C co-precipitated the motor protein dynein but not kinesin (panel 2f and 2g).

In all cases the control precipitate (panel 2a-2i, con), obtained from Strep-precipitation with mock transfected lysates, did not exhibit the corresponding signal, indicating no or only low unspecific binding.

To further validate the association of HDGF-C with tubulin and dynein, co-immuoprecipitation with endogenously expressed HDGF-C was performed. HepG2 cells were lysed, and cleared lysates were incubated with HDGF-C antibody. Immunoprecipitation was performed by using protein A sepharose beads. Obtained samples were analysed by subsequent Western blot and the results are shown in figure 21. Similar experiments for HDGF-B and HDGF-A could not be performed due to the missing of appropriate antibodies.
Results

**Figure 21: Co-Immunoprecipitation of HDGF-C** Co-immunoprecipitation was performed from cleared HepG2 lysates using the HDGF-C antibody as described in the method section. Obtained immunoprecipitates (IP C), pre-clearing (PC) and 10 µg anti HDGF-C antibody (Ab) were separated on 12 % SDS PAGE gels, transferred to PVDF membranes. Membranes were incubated with anti-dynein (panel 1), anti-tubulin (panel 2) or goat anti pan-HDGF (panel 3). The asterisk (*) indicates the co-precipitated tubulin. Molecular mass marker lanes are indicated on the left.

The results of the immunoprecipitation from an endogenous expressing source (HepG2 cells) (figure 21) supported the previous result obtained with ectopically expressed protein (figure 20). Endogenous HDGF-C was able to co-precipitate dynein (panel 1) as well as tubulin (panel 2). Due to the similar running behaviour of heavy chain and tubulin, the tubulin signal (marked with an asterisk) could not be further separated from the heavy chain signal. Pre-cleared samples did not show any corresponding signal, indicating that there was not any unspecific binding to the protein A beads.

Since HDGF-A was not able to co-precipitate dynein or tubulin (figure 20), this result confirmed once more, that the HDGF-C specific antibody did precipitate HDGF-C.

**3.2.3.2.2 N-terminal peptide of HDGF-A determines interaction behaviour**

As shown in the previous chapter, HDGF-B and HDGF-C exhibited a quite different interaction behaviour compared to that of HDGF-A. Nucleolin and YB-1, which interact with HDGF-A, were not co-precipitated by HDGF-B or HDGF-C. In contrast, the co-precipitated tubulin and dynein could not be co-precipitated by HDGF-A.
The alignment of HDGF isoforms (figure 9) revealed that there were not any obvious similarities between the altered N-termini of HDGF-B and HDGF-C and moreover, similarities were only present in that part, which is also present in HDGF-A. From this point of view it is surprising that HDGF-B and -C interacted with the same proteins, although these particular proteins could not be found in the interactome of HDGF-A. In addition, with regard to the interacting proteins of HDGF-A, they also lost the same interaction partners.

However, both miss the first N-terminal amino acid residues of HDGF-A. Therefore, the differences in the interaction pattern to HDGF-A might depend rather on the loss of the respective peptide as on the altered N-termini. To study this, a truncated HDGF-A variant, deleted in the first N-terminal 15 amino acid residues (NΔ15) [33] was subjected to StrepTactin precipitations as described before. Obtained results are shown in figure 22, panel 1.

**Figure 22:** N-terminal peptide determines interaction behaviour of HDGF. HepG2 cells were transiently transfected with plasmids encoding C-terminal Strep-tagged HDGF-A, HDGF-B and HDGF-C; an N-terminally truncated HDGF-A variant (NΔ15), which was deleted in the first 15 N-terminal amino acid residues, as well as constructs containing the first 30 (N30), 15 (N15), 10 (N10) N-terminal amino acid residues of HDGF-A followed by a SNAP-Strep sequence (see section 2.2.7). Cleared lysates were subjected to StrepTactin co-precipitation. Eluates were analysed by Western blot using antibodies against nucleolin (panel 1a & 2a), YB-1 (panel 1b), tubulin (panel 1c), pan-HDGF (panel 1d) and Strep-tag (panel 2b). Marker lanes are indicated on the left.
Strikingly, the co-precipitates of NΔ15 HDGF-A variant (figure 22) resembled that of HDGF-B and HDGF-C (figure 20). NΔ15 HDGF-A lost almost completely its ability to co-precipitate nucleolin (panel 1, 1a) and YB-1 (panel 1, 1b). By contrast, it was capable to co-precipitate tubulin (panel 1d).

Consequently, deleting the first 15 amino acid residues of HDGF-A was sufficient to change the interaction pattern referring to tubulin, YB-1 and nucleolin. However, the first N-terminal amino acid residues of HDGF-A were not sufficient to mediate interaction to nucleolin or Yb-1 alone, as shown in figure 22, panel 2. Corresponding eluates of the constructs, containing either the first 10 (N10), 15 (N15) or the first 30 (N30) amino acid residues of HDGF-A, did not exhibit a signal for the mentioned proteins.

3.2.3.2.3 RNA dependent interaction of HDGF-A to nucleolin and YB-1

The interaction of HDGF-A and nucleolin depends on RNA association [123]. Like nucleolin YB-1 has RNA binding properties and participates in RNA related processes such as transcription and splicing or regulation of mRNA stability [170]. Furthermore, in both cases the outermost N-terminal amino acid residues of HDGF-A were essential for the association as shown in figure 22 and in addition, the novel isoforms HDGF-B and -C failed to co-precipitate both proteins.

Due to these parallels between nucleolin and YB-1, the association of YB-1 and HDGF-A might follow a similar RNA dependent interaction pattern as observed for HDGF-A and nucleolin. To test this, the protocol from Bremer et al. [123] was adapted by which lysates from Strep-tagged HDGF-A expressing cells were pre-treated with RNase A followed by StrepTactin co-precipitation. Resulting eluates were analysed by Western blot for the presence of YB-1 as well as nucleolin, which served as positive control. Corresponding results are shown in figure 23.
Results

Figure 23: RNA dependent interaction of HDGF-A and YB-1. HepG2 cells were transiently transfected with C-terminally Strep-tagged HDGF-A. Cleared lysates were incubated in the presence as well as in the absence of RNase A over night followed by StrepTactin precipitation as described under section 2.1.9.5. Obtained precipitates were analysed by Western blot using anti-YB-1 (panel 1), anti-nucleolin (panel 2) or anti Strep-tag antibody (panel 3). Molecular mass marker lanes are indicated on the left.

Pre-treatment with RNA led to an apparent loss of co-precipitated YB-1 (figure 23, panel 1) as previously shown for nucleolin [123] (panel 2). This result strongly suggested that YB-1 – HDGF-A complex formation depended also on the presence of RNA.

3.2.3.2.4 HDGF-C and its co-localisation with other proteins

To investigate further the putative interaction of HDGF-C with elements of the cytoskeleton (figure 20 & figure 21), immunofluorescence analysis was performed to observe potential co-localisations of endogenously expressed HDGF-C with some of the putative interaction partners. Therefore, human dermal fibroblasts were fixed and permeabilized as described in the method section (2.1.4). Cells were treated with HDGF-C specific antibody in combination with further antibodies, directed against following proteins of interest: nucleolin, Yb-1, tubulin, actin, vimentin, kinesin and dynein. Obtained images are represented in figure 24.
Figure 24: Co-localisation of HDGF-C with dynein and tubulin. For immunofluorescence analysis human dermal fibroblast were fixed and permeabilized as described in the method section. Cells were incubated with anti HDGF-C (panel I A-H, red) in combination with monoclonal anti-HDGF H3 (panel II B, green), anti-nucleolin, (panel II B, green) anti-YB-1 (panel II C; green), anti-actin (panel II D, green), anti-vimentin (panel II E, green), anti-tubulin (panel II F), anti-dynein (panel II G, green) and anti-kinesin (panel II H, green). Cells were counterstained with DAPI (panel III, A-H). Panel IV (A-H) shows the superposition of the red, blue and green signals. Insets display zoomed views of the indicated area. Scale bars: 10 µm (right corner).
HDGF-C was detected in nucleus as well in the cytoplasm of cells (figure 24, panel I, A-H). Its endogenous appearance resembles that obtained after ectopically overexpression (figure 12). However, the dominant nuclear staining as observed after overexpression was not that pronounced in the images of endogenous HDGF-C. The cytoplasmic staining revealed granules-like structures. Staining with a monoclonal pan-HDGF antibody (H3) resulted in a predominant nuclear staining and very weak cytoplasmic staining (panel II, A), which is in line with the observation of numerous other studies [3, 17, 29]. Considering the huge dominance of HDGF-A towards HDGF-B and HDGF-C in dermal fibroblasts (figure 9), the monoclonal pan-HDGF antibody detected most probably mainly HDGF-A in the specimen. Nevertheless, co-localisation of signals obtained with isoform specific antibody and pan-HDGF antibody could be observed mainly in the nucleus (panel IV, A).

According to the results obtained from co-precipitations (figure 20) HDGF-C is able to interact with the cytoskeleton proteins actin, vimentin and tubulin and not with the nuclear proteins Yb-1 and nucleolin. As expected, a co-localisation of HDGF-C with YB-1 (figure 24 panel IV, C) or nucleolin (panel IV, B) was not detected. Co-staining of HDGF-C with actin and vimentin revealed that HDGF-C did not really co-localise with vimentin (panel IV, E) and actin (panel IV, D), although the analysis of the co-precipitates would suggest this. Co-staining with tubulin (panel IV, F) demonstrated that the granule-like structures of HDGF-C superimposed on the tubulin network (panel IV, F). Furthermore, a strong co-localisation was observed when cells were stained with HDGF-C antibody and dynein antibody (panel IV, G). By contrast, the motorprotein kinesin did not co-localise considerably with HDGF-C (panel IV, H) which corresponded to the previous obtained results of the co-precipitations (figure 20).
Results

3.3 The secretion of HDGF

HDGF was originally identified as secreted factor from the conditioned medium of the hepatocellular carcinoma cell line Huh-7 [1]. Also other studies demonstrated that HDGF is present in the secretome of different cell types such as endothelial cells [37], neural stem cells [55], glioblastoma cells [55] or skeletal muscle cells [78]. These data refer to HDGF-A, whether the two novel HDGF isoforms HDGF-B and HDGF-C are secreted is not known.

Proteins secreted via classical secretion pathway require an N-terminal signal peptide leader sequence, which spans the first N-terminal 16-30 amino acid residues [90, 173]. This sequence mediates the transfer into the ER. In this cellular compartment the protein is folded and modified before it is secreted into the extracellular space via exocytosis [174].

A typical secretion signal sequence consists of some positively charged amino acid residues, a central hydrophobic region of 6-12 amino acid residues followed by more polar C-terminal part which contains the cleavage site [173]. The N-terminal sequence of the HDGF isoforms does not contain such a long hydrophobic as depicted in table 8.

Table 8: N-terminal amino acid Sequences of HDGF-A, -B and -C does not contain a signal sequence: The first 33 N-terminal amino acid residues of each isoform are given as single letter code. Hydrophobic/unpolar amino acid residues are coloured red, basic amino acid residues are blue, acidic amino acid residues are green and neutral/polar amino acid residues are black. SignalP 4.1 prediction tool was used with default settings to estimate the probability of signal sequence for the isoforms. The calculated D-score (discrimination score) was in each case below the default cut-off value of 0.45, thus, sequences did not contain signal peptides.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>N-terminal Sequence</th>
<th>D-score</th>
<th>Signal?</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDGF-A</td>
<td>MSRSNQRKEYKCGDLVFAKMKGYPHWPARIEM33</td>
<td>0.107</td>
<td>no</td>
</tr>
<tr>
<td>HDGF-B</td>
<td>MHPEGGQFVPQLGHTLAKLRFFLSKGGRA33</td>
<td>0.272</td>
<td>no</td>
</tr>
<tr>
<td>HDGF-C</td>
<td>MEQRAGGNRVQTSTLCAGAAVIDEMPEAAVKS33</td>
<td>0.126</td>
<td>no</td>
</tr>
</tbody>
</table>

The analysis of the three HDGF sequences by the use of the prediction tool SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) [175], an online tool which is able to predict signal sequences in proteins, supported the notion. The discrimination-scores (D-score) obtained with SignalP 4.1 were 0.107 (HDGF-A), 0.207 (HDGF-B) and 0.126 (HDGF-C) deceeding considerably the default cut-off value of 0.45. Therefore, HDGF isoforms are not likely secreted via the ER/Golgi pathway. The corresponding Signal IP outputs are shown in the appendix (A1-A3).
3.3.1 Time dependent secretion of HDGF isoforms

Thakar et al. demonstrated that the first 11 amino acid residues of HDGF-A play an essential role in the unconventional HDGF-A release. N-terminally truncated HDGF-A proteins, missing this specific peptide sequence, were not present in the conditioned media 24 h after ectopic overexpression while the full length form can be detected at the same time [33]. Interestingly, N-terminal truncation of the first 12-21 amino acid residues (exact number could not be determined) can be induced by exchanging the putative phosphorylation site Ser165 to an Ala [33]. HDGF-B and HDGF-C do not possess this HDGF-A specific N-terminal peptide, which mediates the secretion of HDGF-A. This led to the question whether HDGF-B and HDGF-C are secreted or whether they remain in the cell like observed for other truncated HDGF-A forms [33].

In order to address this question HepG2 cells were transiently transfected with respective plasmids encoding HDGF-A, HDGF-B and HDGF-C as well as with the Ser165 to Ala mutant of HDGF-A, termed HDGF-A S165A in the following. The conditioned media was collected to different time points (8 h, 20 h, 26 h, 30 h, 44 h after transient transfection) and acetone precipitated to concentrate the protein content. In order to detect the presence of HDGF variants these samples were subjected to Western blot analysis. The results of Western blot analysis are shown in figure 25.

Proteins can also be released passively when cells lose their membrane integrity as a consequence of cell death. In order to exclude this possibility, extracellular LDH activity was monitored in each supernatant as indicator for leakage [120]. The corresponding LDH values are given below the corresponding Western blots (figure 25). In all cases the activity was below 5 %, indicating that the loss of membrane integrity was low.

Since a goat anti pan-HDGF antibody was used, there was of course the possibility of background staining due to the presence of endogenous HDGF. However, HepG2 cells did not release endogenously expressed HDGF isoforms in such amounts, that it was detectable in Western blot analysis. Therefore, background staining caused by endogenous HDGF could be excluded in the supernatant samples.
**Figure 25: Secretion of HDGF isoforms and HDGF-A S165A.** HepG2 cells were transiently transfected with plasmids encoding HDGF isoforms HDGF-A (panel 1), HDGF-B (panel 2), HDGF-C (panel 3) or the HDGF-A S165A (panel 4). Over the course of time supernatants (SN) were collected from the transfected cells at the indicated time points (8 h, 20 h, 26 h, 30 h, 44 h). Finally cells were lysed. Supernatants (SN) (panel I) and cell lysates (CL) (panel II) were separated on 12 % SDS PAGE gels and transferred to PVDF-membranes. Membranes were probed with goat anti pan-HDGF. Molecular mass marker lanes are indicated on the left. As described under method section 2.1.7 extracellular LDH activity was determined in each collected SN and is given below the corresponding Western blot. Data (given in % of total extracellular LDH) represent the means ± standard deviations for triplicate assays.

HDGF-A could be detected after 20 h incubation time (panel 1), a timeframe that was not sufficient to detect the other HDGF forms in the conditioned media (panel 2-4). Isoform HDGF-B (panel 2) and mutant HDGF-A S165A (panel 4) could not be detected after 44 h either. HDGF-C (panel 3) was present in the incubation medium in detectable amounts after 30 h but signals were weaker compared to HDGF-A.

The analysis of the respective cell lysates demonstrated that all proteins were ectopically expressed (panel II).

### 3.3.2 DMA inhibits secretion of HDGF-A, HDGF-C and endogenous HDGF

Since HDGF-A and HDGF-C did not contain a secretion peptide (table 8) but could be detected in the surrounding media of cells (figure 25), the question remained open how these proteins leave the cell.
Results

Several alternative secretion pathways have been described in literature, which are independent from the ER/Golgi route [92]. To identify the putative secretion pathway, initial experiments were performed using different inhibitors that interfere with different alternative secretion pathways (Kappelmann, unpublished data). Interestingly, when transiently transfected Cos7 cells were treated with DMA, recombinant HDGF-A release was significantly decreased.

The drug DMA inhibits Na⁺/H⁺ exchanger and Na⁺/Ca²⁺ exchanger [176]. Furthermore, it was reported that treatment of cells with DMA reduces the release of exosomes probably caused by a decrease of intracellular Ca²⁺ levels [177].

To verify the effect of DMA on HDGF-A secretion and to see whether the drug DMA inhibits also the secretion of ectopically expressed HDGF-C, similar inhibitor experiments were performed in the present work. Since ectopically expressed HDGF-B and HDGF-A S165A could not be detected in the surrounding media after 40 h (figure 25, panel I.2 & I.4, respectively), these proteins were excluded from the following inhibitor experiments. HepG2 cells were transiently transfected with a plasmid encoding HDGF-A, HDGF-C or Luciferase-Strep, which served as control protein in this experiment. The gaussia Luciferase-Strep construct contained an artificial classical N-terminal signal peptide and therefore was released from cells into the extracellular space by the ER/Golgi driven secretion pathway.

Furthermore, the effect of DMA on endogenously expressed HDGF isoforms was examined. Since endogenous HDGF could not be found in the surrounding media of HepG2 cells, this cell type was inappropriate for this experiment. Instead, HeLa cells were selected because prior experiments revealed that endogenous HDGF could be detected in their conditioned media after 40 h incubation.

As mentioned before, the new identified isoforms have a similar molecular weight and a similar running behaviour in Western blot analysis. For the following analysis a goat anti pan-HDGF antibody was used, which is able to recognize all three HDGF isoforms (figure 11). Consequently, the following analysis did not reveal which endogenous HDGF isoform was detected in the supernatants from HeLa cells. Therefore, in these cases the term “HDGF” was used and the isoform A/B/C was not further specified.

To exclude the possibility that HDGF proteins were passively released into the extracellular space, the extracellular LDH activity was determined simultaneously. Values are given below the respective Western blots images (figure 26).
Results

Figure 26: Inhibitory effect of DMA on HDGF Secretion. HepG2 cells were transiently transfected with plasmids encoding HDGF-A (panel 1), HDGF-C (panel 3) or control protein Luciferase-Strep (panel 2) with an additional N-terminally added secretion signal peptide. 4 h after transfection, serum was changed to serum free medium and cells were incubated in the presence of the indicated inhibitors (see methods section), or with respective solvents DMSO or EtOH; con (control, no treatment). In case of endogenous HDGF, HeLa cells were incubated (panel 4) in medium containing DMA or the solvent DMSO. After 24 h (HDGF-A; Luciferase-Strep) or 42 h (HDGF-C; non-transfected HeLa cells) supernatants (SN) were collected. Cleared cell lysates (CL) and acetone precipitated SN were analysed by Western blot using anti pan-HDGF antibody. Molecular mass marker lanes are indicated on the left. As indicator for cell viability extracellular LDH activity was determined in each supernatant. Values from triplicate measurements are given as mean in % of total LDH ± standard deviation.

DMA treatment decreased the release of ectopically expressed HDGF-A (figure 26, panel 1a). Furthermore, Western blot analysis of corresponding total cell lysates (panel 1b) demonstrated that transfection was successful in each case and protein levels were in a similar range.
In contrast to DMA, Monensin (Mon), a well known inhibitor of the ER/Golgi mediated secretion pathway [178], did not result in lower amounts of extracellular HDGF-A (panel 1a). Indeed, Mon even increased the level of extracellular HDGF-A. At the same time the corresponding solvent of Mon ethanol (EtOH) did not influence secretion levels of HDGF-A and the detected extracellular HDGF-A signals were comparable to that of the control. In each case the intracellular protein level remained also here constant, as shown by the Western blot analysis of the cleared lysates (panel 1b). The measured extracellular LDH activity (given below the corresponding Western blots) was in a similar low range, indicating that the putative passive release was comparably low in each sample.

In order to confirm that DMA did not influence secretion of proteins, which were exported via the classical secretion pathway, HepG2 cells were transiently transfected with a plasmid encoding Strep-tagged *gaussia* Luciferase (panel 2). The Western Blot analysis of the supernatants (panel 2a) demonstrated that Mon was indeed able to inhibit the ER/Golgi driven secretion of Luciferase-Strep, the corresponding signals were considerably lower compared to the control sample. In contrast to this, DMA treatment did not reduce Luciferase secretion and the corresponding protein signal was comparable to that of the control. At the same time inhibitor treatment did not have any significant impact on the intracellular protein levels (panel 2b).

Like HDGF-A secretion also HDGF-C secretion could be decreased by treating the transiently transfected cells with DMA. The respective Western blot (panel 3a) showed that the signal was weaker compared to that of the control. Simultaneously, the solvent DMSO did not reduce secretion of HDGF-C. Worthy of note, the DMA-induced decrease of HDGF-C release was not as strong as observed for HDGF-A.

Also the effect of Mon on HDGF-C release was studied. But majority of cells died after 40 h incubation in presence of this inhibitor, leading to unreliable results. Thus, this experiment was excluded from the results.

In order to see whether DMA is able to decrease the secretion of endogenous HDGF, HeLa cells were incubated in presence of DMA or DMSO for 40 h (non-transfected, panel 4a). DMA treatment reduced HDGF release. Hence, observed DMA effect was not an artificially induced phenomenon only observed for transiently over-expressed HDGF proteins. However, due to longer incubation times the extracellular LDH values were slightly increased in these samples.
3.3.3 The presence of HDGF in extracellular vesicles

3.3.3.1 Presence of HDGF isoforms and HDGF-A S165A in extracellular vesicles

The inhibitory studies demonstrated that DMA treatment led to a reduction of HDGF-A, HDGF-C and of endogenous HDGF release (figure 26). Since DMA was shown to decrease exosome release [177], the observed effect provided indication that those proteins might be released via the exosome mediated pathway.

Exosomes are commonly isolated on basis of their size by a centrifugation protocol involving several centrifugation steps at different velocities [105]. The respective protocol is depicted in figure 27 (panel I). It is necessary to consider that the separation is not leading to an absolutely pure exosome fraction. Apoptotic blebs and shedding microvesicles, both extracellular vesicles which derive from the plasma membrane and not from the endosomal compartment, can be still present. In general these vesicles are greater than exosomes and therefore sediment already at velocities of 10 000 x g. But since apoptotic blebs and shedding microvesicles are very variable in size and shape, it is possible that subfractions of smaller size are present, which does not sediment at 10 000 x g and therefore can be found in the exosome fraction [99, 179].

In order to examine whether HDGF-A and HDGF-C are present in exosome-enriched fraction, HepG2 cells were transiently transfected with plasmids encoding respective HDGF constructs and isolated the corresponding exosomes. Although HDGF-B and the HDGF-A mutant HDGF-A S165A could not be identified in the conditioned media, these variants and the possible presence in extracellular vesicles were studied as well. Samples were analysed by Western blot analysis using pan-HDGF antibody and antibodies against exosomal proteins CD63 and actin. Results are shown in figure 27 (panel II).
Results

Figure 27: HDGF isoforms in exosome-enriched fractions. HepG2 cells were transiently transfected with plasmids encoding HDGF isoforms HDGF-A, HDGF-B, HDGF-C and HDGF-A S165A. After 40 h incubation in serum free medium, cell culture medium was collected. Exosomes were enriched as described under method section 2.1.8.1 and as depicted in panel I. During each centrifugation step aliquots of the supernatants were collected (SN1-SN5) and concentrated by acetone precipitation. SN1-SN5 and exosome-enriched fraction (Ex) were separated on 12 % SDS PAGE gels, transferred on PVDF membranes and probed with goat anti pan-HDGF (panel II 1a, 2a, 3a, 4a), anti-CD63 (panel II 1b, 2b, 3b, 4b) or anti-actin (panel II 1c, 2c, 3c, 4c) as indicated on the right. Molecular mass marker lanes are shown on the left.

HDGF-A could be detected in all supernatants as well as in the exosome-enriched fraction (figure 27, panel II, 1a). During the centrifugation protocol supernatants were collected (SN1- SN5) after each centrifugation step. SN5 was that fraction, which was collected after the sedimentation of exosomes as depicted in panel I. Consequently, SN1-SN4 contained exosomes. However, a significant decrease in the HDGF-A signal intensity due to a putative exosome depletion in SN5 was not observe. Furthermore, the HDGF-A signal in the exosome fraction was significantly weaker compared to those in the concentrated supernatants.
CD63, a family member of the tetraspanin family, is highly abundant in exosomal membranes [107]. Due to this CD63 immunodetection served as positive control for the presence of exosomes. The core protein CD63 has a molecular weight of 26 kDa, due to high glycolysation the actual molecular weight ranges from 30-60 kDa [180, 181]. The immunodetection demonstrated that CD63 was highly enriched in the exosome fraction (panel II, 1b). While a strong signal in the exosome fraction was present, it was nearly completely absent in the corresponding supernatants. Hence, this signal distribution of CD63 between supernatants and exosome enriched fraction formed a considerable contrast to that obtained for HDGF-A.

In addition membranes were probed with anti-actin antibody (panel II, 1c). In SDS PAGE gels actin runs at a molecular weight position of approximately 40 kDa. Since the cytoskeleton protein actin is also a common component in exosomes [182, 183], the detection of actin served as further positive control for the presence of exosomes. Compared to CD63, actin was not enriched in the exosome fraction to the same extent.

Most strikingly, HDGF-B (panel II, 2a) and the mutant HDGF-A S165A, (panel II, 4a) were heavily enriched in the final exosome fraction. Both were below detection limits in the corresponding supernatants. Consequently the signal distribution was comparable to that of the exosomal marker protein CD63, shown in panel 2b and 4b, respectively. While Western blot analysis of the total cell lysates resulted in a triple signal for HDGF-B, detected at a molecular weight position between 35 and 40 kDa in SDS PAGE gels (figure 11), corresponding exosome fractions comprised only double signals of a comparable molecular weight range.

Like HDGF-A, HDGF-C was detectable in the surrounding media and in the respective exosome containing fraction. Interestingly, in the exosome fraction a second weaker signal became visible at a lower molecular weight position of approximately 35 kDa, which was not detected in the supernatants and in cell lysates (figure 11). This suggested that this smaller protein variant was enriched in the exosome fraction, which corresponded more to the results of HDGF-B and HDGF-A S165A.

### 3.3.3.2 Endogenous HDGF in extracellular vesicles

Since overexpression of proteins is always afflicted with artefacts, studies under physiological conditions are necessary. Therefore, several different human cell lines were selected. Incubation media were collected after 40 h from HepG2 cells, HaCat cells, HeLa cells as well as from the two breast cancer cell lines MCF-7 and MDA-MB-231. Exosome fractions were obtained by stepwise centrifugation, as described before. At every centrifugation step an aliquot of the supernatant (SN1-SN5) was taken. Final
Exosome enriched fraction and supernatants were examined by Western blot analysis. Results are shown in figure 28.

Figure 28: Endogenous HDGF in exosome containing fractions. Panel I: HaCat cells (panel 1), HeLa cells (panel 2), MDA-MB-231 cells (panel 3), MCF-7 cells (panel 4) and HepG2 cells (panel 5) were incubated in serum free medium for 40 h. Total lysates (CL), supernatants (SN) and corresponding exosome fraction (Ex) were collected from the indicated cell lines and were analysed by Western blot using rabbit anti pan-HDGF (panel I: 1a, 2a, 3a, 4a, 5a), anti-CD63 (panel I: 1b, 2b, 3b, 4b, 5b) or anti-actin (panel 1c, 2c, 3c, 4c, 5c) as indicated on the right side. Molecular mass marker lanes are shown on the left. Panel II: Dynamic light scattering (DLS) measurement of cell type specific exosome fractions and extracellular LDH activity (%) measured at the time of collection. LDH measurement was performed in triplicates; values are given in % of total LDH ± standard deviation. DLS was recorded with 6 replicate measurements. Mean values are given ± standard deviation.

Furthermore, corresponding extracellular LDH activity was determined as indicator for cell leakage (figure 28, panel II).
To characterise the obtained final exosome containing fraction dynamic light scattering (DLS) analysis was performed. DLS analysis revealed the presence of particles with a hydrodynamic diameter of 100-150 nm (panel II, 6). This increased values corresponded to the reported size of exosomes obtained in DLS measurements [184].

Interestingly, secretion of HDGF is cell-type specific. Western Blot analysis demonstrated that endogenous HDGF could be detected in supernatants and exosome fraction collected from HaCat cells (figure 28, panel 1a) HeLa cells (panel 2a) and MCF-7 (panel 4a). Endogenous HDGF was below detection levels in supernatants collected from MDA-MB-231 (panel 3a) and HepG2 cells (panel 5a) although analysis of respective total cell lysates demonstrated that all examined cell lines expressed HDGF at comparable levels (figure 29, panel 2a).

![Figure 29: Comparison of endogenous HDGF in exosome containing fractions.](image)

A further interesting observation was the identification of two HDGF polypeptides and their diverse distribution in different samples. The goat anti pan-HDGF antibody detected two polypeptides at a position of 37 kDa and 39 kDa in the supernatants of HaCat cells, the smaller form being predominant in all five supernatants. In the corresponding HaCat exosome fraction the 39 kDa protein signal was as strong as the 37 kDa signal. This clear enrichment of the 39 kDa signal in exosome fraction compared to the supernatants could not be observed in the samples of the other cell types. In supernatants and exosome-enriched fractions of HeLa and MCF 7 cells the higher 39 kDa molecular weight form appeared only as weak signal. HDGF was also absent in
the collected exosomes from HepG2 cells while in exosomes from MDA-MB-231 only a weak 39 kDa signal was detectable.

It is important to note, that in cell lysates the 37 kDa signal is by far the dominant band whereas the 39 kDa signal is hardly detectable.

3.3.3.3 HDGF-A and endogenous HDGF are present in vesicle-depleted supernatants

SN5, collected after sedimentation of exosomes (figure 27, panel II), still contained detectable amounts of HDGF-A and HDGF-C (figure 27, panel II). A similar picture was obtained for endogenous HDGF, released from the different cell types (figure 28). On the contrary, exosomal marker CD63 as well as HDGF-B and HDGF-A S165A could not be detected in the SN5. This observation suggests that only a fraction of HDGF-A or -C is vesicle-associated, whereas HDGF-B and HDGF-A S165A are exclusively in the vesicle fraction.

To address this, SN5 was subjected to an additional ultracentrifugation at 112 000 x g for 24 h (figure 30, panel I). According to Théry et al., this timeframe should be sufficient to deplete the remaining exosomes [99]. The obtained supernatant was labelled as SN5* in the following. The samples were analysed by Western blot using goat anti pan-HDGF antibody or anti-CD63 antibody, respectively. Results are shown in figure 30 (panel 2).
Results

Figure 30: Recombinant HDGF-A and endogenous HDGF are still present in vesicle-depleted supernatants. Panel I: After 40 h incubation supernatants were collected and subjected to stepwise centrifugation to isolate exosomes as described before. SN5, collected after 120 000 x g step, was centrifuged a second time for 24 h at 112 000 x g to deplete the supernatant completely from extracellular vesicles (SN5*). Panel II: SN4, SN5* and exosome fraction (Ex) were collected from HDGF-A/B/C (panel II 1-3) or HDGF-A S165A (panel 4) overexpressing and from non-transfected (nt) HepG2 cells (panel 5) or nt HeLa cells (panel 6). Samples were analysed by Western blot using anti pan-HDGF (panel 1-5) or anti-CD63 antibodies (panel 6) as indicated on the right. Marker lanes are shown on the left.

In fact exosomal marker CD63 was not detected in this supernatant obtained after 24 h ultracentrifugation at 112 000 x g, indicating that the majority of exosomes were sedimented at this step (figure 30, panel II 6). A similar picture was observed for HDGF-B (panel II 2), HDGF-C (panel II 3) and HDGF-A S165A (panel II 4). In each case the recombinant protein was not detectable in the final, exosome-depleted supernatant SN5*.

By contrast, ectopically overexpressed HDGF-A (panel II 1) as well as endogenous HDGF (panel II 5), released from HeLa cells, were still present in this exosome-depleted supernatant SN5*. Interestingly, in both exosome-depleted supernatants SN5* a significant decline of protein compared to SN4 (collected after 15 000 x g) could be observed, indicating that only parts remain in the exosome-depleted supernatants while the remaining subfraction can be sedimented in the final exosome enriched fraction. In agreement with the observation of this decline is the presence of these proteins in the exosome containing-fraction. In this context it is necessary to consider that the proteins are concentrated to different amounts in Ex fraction and corresponding SN fraction.
3.3.3.4 Localisation in or on extracellular vesicles

Exploring the localisation of HDGF in or on extracellular vesicles will provide more insight in the role of the delivered cargo. Proteins on the surface might be involved in communication processes between extracellular vesicle and recipient cell or can be integrated in the plasma membrane by fusion of vesicles with recipient cells. Proteins in the lumen of vesicles can enter the cytoplasm of cells when extracellular vesicles are internalised and elicit inside different cell responses [185].

To clarify the localisation of HDGF inside or outside of extracellular vesicles a stripping assay was applied, which was used by several other groups [186, 187]. Obtained exosome containing fractions were treated with the protease trypsin in presence or absence of the detergent Triton X-100. Trypsin treatment alone can digest proteins on the vesicle surface but proteins within vesicles are not affected due to the surrounding vesicle membrane. Treating vesicles with the detergent Triton X-100 leads to the lysis of the membrane, without affecting the vesicle associated proteins. Only the combined treatment of detergent and trypsin leads to the digestion of intravesicular proteins.

3.3.3.4.1 Localisation of HDGF isoforms and HDGF-A S165A in extracellular vesicles

First, HepG2 cells were transiently transfected with plasmids encoding HDGF-A, HDGF-B and HDGF-C and HDGF-A S165A. Exosome containing fractions were collected and subjected to the described procedure. The obtained fractions were analysed by Western blot using either goat pan-HDGF antibody or anti-actin antibody. According to Théry et al. actin is localised in the lumen of exosomes [105]. Consequently, the detection of actin in those samples that were exposed to trypsin, demonstrated the presence of intact vesicles. The corresponding results are shown in figure 31.
**Results**

HDGF-A was resistant to trypsin treatment in the absence of Triton X-100 (figure 31, panel 1), indicating that this protein was located in the lumen of extracellular vesicles like the control protein actin. Combined treatment of Triton X-100 and trypsin resulted in the complete loss of HDGF-detection. This demonstrated that the trypsin protease was functional under the used conditions. Interestingly, in contrast to HDGF-A, HDGF-B (panel 2), HDGF-C (panel 3) and HDGF-A S165A (panel 4) were sensitive to trypsin digest in the absence of the detergent. The corresponding immunodetection of actin demonstrated that in all cases vesicles remained intact. Therefore, this experiment suggested that these alternative isoforms and the mutant were located on the surface of extracellular vesicles, where they were accessible to trypsin treatment.

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**Figure 31: Localisation human HDGF-A, -B, -C and HDGF-A S165A in extracellular vesicles.**

Exosome enriched fractions were collected from HepG2 cells transfected with plasmids encoding HDGF-A (panel 1), HDGF-B (panel 2) or HDGF-C (panel 3) or HDGF-A S165A (panel 4). As described under Methods, aliquots of freshly isolated extracellular vesicles were subjected either to mock conditions without treatment of the vesicles, only trypsin digestion, only Triton X-100 lysis or Triton X-100 lysis prior to trypsin digestion. Samples of all four conditions were separated on 12 % SDS-gels and transferred to PVDF membranes. Membranes were immunoblotted with goat anti-pan-HDGF (panel a), followed by anti-actin (panel b) as indicated on the right side. Marker lanes are shown on the left.
A similar result was obtained also for mouse HDGFwt and mHDGF S165A as shown in figure 31. As mentioned before, in the mouse genome isoform HDGF-B and isoform HDGF -C does not exist.

![Figure 32: Localisation of murine HDGF wt and HDGF S165A in extracellular vesicles.](image)

Exosome containing fractions were collected from Hek293 cells transfected with plasmids encoding murine HDGF wt (panel 1) or murine HDGF S165A (panel 2). As described under Methods, aliquots of freshly isolated extracellular vesicles were subjected either to mock conditions without treatment of the vesicles, only trypsin digestion, only Triton X-100 lysis or Triton X-100 lysis prior to trypsin digestion. Samples of all four conditions were separated on 12 % SDS gels and transferred to PVDF membranes. Membranes were immunoblotted with anti-mouse HDGF (panel a), followed by anti-actin (panel b) as indicated on the right side. Marker lanes are shown on the left.

This result indicated that the phenomenon of alternative vesicular localisation, induced by the exchange of serine residue 165, is not species specific or more precisely at least available for mouse and human HDGF.

While murine HDGF was not digested in the presence of trypsin (panel 1), the respective mutant HDGF S165A (panel 2) were susceptible to the trypsin exposure.

Murine HDGF appeared as double signal in Western blot analysis. The reason is an unusual caspase cleavage site in murine HDGF [188].

### 3.3.3.4.2 Vesicular localisation of endogenously expressed HDGF

The same experiment was performed with exosome containing fractions obtained from HeLa, HaCat and MCF-7 cells. Since MDA-MB-231 and HepG2 cells did not release endogenous HDGF in detectable amounts (figure 28 & 29), these cell lines were not
included in this experiment. The results of the Western blot analysis are depicted in figure 33.

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1. HaCat
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b) 35

2. HeLa
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b) 35

3. MCF-7
a) 40
b) 35

Figure 33: Localisation of endogenous HDGF in extracellular vesicles. Exosome containing fractions were collected from the indicated cell lines HaCat (panel 1), HeLa (panel 2) or MCF-7 (panel 3). As described under Methods, aliquots of freshly isolated extracellular vesicles were subjected either to mock conditions without treatment of the vesicles, only trypsin digestion, only Triton X-100 lysis or Triton X-100 lysis prior to trypsin digestion. Samples of all four conditions were separated on 12 % SDS gels and transferred to PVDF membranes. Membranes were immunoblotted with rabbit anti pan-HDGF (panel 1a, 2a, 3a), followed by immunodetection with anti-actin (panel 1b, 2b, 3b) as indicated on the right side. Marker lanes are shown on the left.

The Western blot analysis, shown in figure 33, demonstrated that endogenous HDGF was resistant to trypsin digest in the absence of the detergent Triton X-100. This provided evidence that for all tested cell types endogenous HDGF is located predominantly inside of extracellular vesicles, where it is protected from trypsin degradation. Combined treatment with Triton X-100 and trypsin led to a complete degradation.

3.3.3.4.3 Phosphorylation mutants and their localisation in extracellular vesicles

The finding that the exchange of one single phosphorylation site Ser165 to Ala led to alternative localisation on extracellular vesicles prompted to the question whether the exchange of other putative phosphorylation sites in HDGF have a similar effect on the presence in extracellular vesicles. Apart from serine residue at position 165 [48], HDGF
contains several other putative phosphorylation sites, which are experimentally confirmed such as Ser132 and Ser133 [48] or Ser103 [36]. In contrast to S165A mutant, all these mutants are released in the conditioned media [33].

Figure 34: Localisation of phosphorylation mutants in extracellular vesicles. HepG2 cells were transiently transfected with plasmids encoding different HDGF mutants either with single Ser to Ala exchanges (panel 2: S132A; panel 3: S133A), with double Ser to Ala exchanges (panel 1: S102/S103A; panel 4: S132/S133A; panel 5 S132/S165A) or with triple exchanges (panel 6 S132/S133/S165A) as indicated above. Panel I: Exosome fractions were collected from conditioned media as described under Methods and corresponding cells were lysed. Aliquots of Ex were left either untreated or incubated with trypsin, with 1% Triton X-100 or with 1% Triton X-100 followed by trypsin treatment as described under Methods. Samples of all four conditions (panel I) and cleared lysates (CL) (panel II) were separated on 12 % SDS PAGE gels and transferred to PVDF membranes. Membranes were probed with anti-mouse HDGF (panel I a-6a and panel II) or anti-actin (panel 1b-6b). Molecular mass marker lanes are indicated on the left.
HepG2 cells were transiently transfected with plasmids encoding different mouse HDGF mutants with different Ser to Ala exchanges. Exosomes were collected and subjected to the protease treatment as described beforehand. Obtained fractions were analysed by Western blot, the respective results are shown in figure 34.

Remarkably, while HDGF-A S165A was sensitive to trypsin digest in absence of Triton X-100 (figure 31), the double Ser to Ala mutant S132/S165A (figure 34, panel I.5) and the triple mutant HDGF S132/133/165A (figure 34, panel I.6) were resistant to trypsin digest. This provided evidence that these HDGF mutants were differentially located in extracellular vesicles. HDGF-A S165A was located at the surface of vesicles. The respective double and triple mutants of HDGF appeared to be within extracellular vesicles. Consequently, the alternative vesicular localisation, caused by the exchange of the Ser165, was abolished when Ser132 and/or Ser133 were additionally exchanged to an Ala in HDGF.

Furthermore, while the exchange of the Ser132 (panel I.2) did not lead to any noticeable difference compared to the wildtype HDGF, mutants with single exchanged Ser 133 (panel I.3) could not be detected in exosome containing fractions anymore. Again only single deletion led to the described effect. Mutants with double (S132/133A) (panel I.4) or triple exchanges (S132/133/165A) (panel I.6) were detected in the exosome containing fractions and moreover like the wildtype form of HDGF they were located within vesicles. The analysis of the respective cell lysates demonstrated that transfection was successful in all cases (panel II).

3.3.3.4.4 N-terminus influences the localisation of HDGF in extracellular vesicles

The present data demonstrated that HDGF-A S165A was not detected in the conditioned media. Nevertheless it could be still identified in the exosome containing fraction (figure 27). Moreover, the present results revealed that this protein is localised at the surface of extracellular vesicles (figure 31). Thakar et al. demonstrated that N-terminal truncation of the first 11 amino acid residues of HDGF is sufficient to block its secretion [33]. This prompted to the question whether truncation of the first N-terminal amino acid residues itself is also sufficient for the observed extravesicular location.

To address this issue murine HDGF constructs, deleted in the first N-terminal 8-12 amino acid residues were transiently overexpressed in HepG2 cells and exosome containing fractions were collected as described before. With respect to localisation on or in extracellular vesicles murine and human HDGF and respective S165A mutants exhibited the same pattern (figure 31 and 32). Therefore, the use of these mouse HDGF constructs should not make a difference in the final result. Isolated exosome containing
fractions were subjected to trypsin in presence or absence of Triton-X 100. The Western blot analysis of the corresponding samples is shown in figure 34.

### I. Triton X-100

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II.

Figure 35: N-terminally truncated HDGF-A mutants are located at the surface of extracellular vesicles. Panel I: HepG2 cells were transiently transfected with plasmids encoding different N-terminal truncated mouse HDGF mutants, deleted in the first 8 (NΔ8) – 12 (NΔ12) amino acid residues. As described under Methods, aliquots of freshly isolated extracellular vesicles were subjected either to mock conditions without treatment of the vesicles, only trypsin digestion, only Triton X-100 lysis or Triton X-100 lysis prior to trypsin digestion. Samples were subjected to Western blot analysis and membranes were probed with anti-mouse HDGF (panel a) or anti-actin (panel b). Marker lanes are indicated on the left. Panel II: Schematic depiction of full length HDGF with highlighting of the first N-terminal 20 amino acid residues. Amino acid residues are given in the single letter code.
While N-terminally truncated mutants, missing not more than 10 amino acid residues (figure 35, panel 1 & panel 2), were not affected by addition of trypsin, the remaining N-terminally truncated HDGF variants, deleted in 11 or more amino acid residues, were sensitive to trypsin digest (panel 3 and 4). The present result clearly demonstrated that the deletion of 11 amino acid residues or more led to a shift from the intravesicular presence to the surface of vesicles.

Thakar et al. figured out, that N-terminally truncated HDGF mutants (NΔ11, NΔ13, NΔ15) cannot be detected in the conditioned media collected after 24 h [33]. Like the NΔ11, NΔ13, NΔ15 HDGF mutants, also HDGF-A S165A and the human isoform HDGF-B were not detected in the conditioned media. Consequently, all these proteins have following features in common: An alternative N-terminus compared to HDGF-A (HDGF-B, HDGF-C) or they missed at least the first N-terminal 11 amino acid residues (HDGF-A S165A, NΔ11); they were not detected in the conditioned media after 24 h incubation time; they were highly enriched in the exosome containing fraction compared to the supernatants and last but not least they were sensitive to trypsin exposure.

Figure 35 (panel II) shows the N-terminal sequence of HDGF. Amino acid residue 11, which seemed to be in the critical position regarding the localisation of the N-terminally truncated HDGF forms (figure 20), is occupied by a Lys. One scenario could be that the presence of this special lysine residue at position 11 is the actual reason why HDGF-A exhibited a complete different secretion behaviour than the other isoform. For example Lys11 could be the target of a posttranslational modification denoting the protein for loading into exosomes.

In order to see whether exchange of single Lys11 have the same effect like observed for N-terminal truncation of HDGF, murine HDGF constructs containing an Arg instead of the Lys at position 8 or 11 were used. Resulting exosome containing fractions were subjected to trypsin treatment in presence or absence of Triton X-100 and samples were analysed by Western Blot. Corresponding results are shown in figure 36.
Results

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1. K8R-Strep

2. K11R-Strep

Figure 36: **HDGF-K8R and -K11R are present within vesicles.** HepG2 cells were transiently transfected with plasmids encoding murine C-terminal Strep-tagged Lys to Arg mutants K8R (panel 1) and K11R (panel 2). As described under Methods, aliquots of freshly isolated extracellular vesicles were subjected either to mock conditions without treatment of the vesicles, only trypsin digestion, only Triton X-100 lysis or Triton X-100 lysis prior to trypsin digestion. Samples were subjected to Western blot analysis and membranes were probed with anti-mouse HDGF. Molecular mass marker lanes are indicated on the left.

Both lysine mutants were resistant to trypsin digest (figure 36). Consequently, both mutants were located within extracellular vesicles displaying the same localisation as the HDGF wildtype. The exchange of Lys11 or Lys8 did not lead to alternative localisation of HDGF. However, since Strep-tagged variants were used in this experiment, the possibility remained that the C-terminal Strep-tag interfered in some way. Due to time constraints untagged lysine mutants could not be tested anymore.

Apart from the Lys 11, the neighbouring Cys at position 12 might be interesting. Thakar et al. showed that this Cys is part of intramolecular disulfide bond which can be formed between Cys12 and Cys108 [33].
Results

Figure 37: HDGF disulfid mutants and their presence in extracellular vesicles. Panel I: HepG2 cells were transiently transfected with plasmids encoding mouse HDGF mutants with an Ala instead of Cys at position 12 and/or 108 (panel 1 C12A; panel 2 C108A; panel 3 C12/108A). Exosome containing fractions were collected from conditioned media. Panel I: Exosome containing fractions were left untreated, incubated with trypsin, treated with 1% Triton X-100 or incubated with Triton X-100 for 30 min at 4°C followed by trypsin treatment. Panel II: Corresponding supernatants SN1-SN5 obtained upon each centrifugation step and exosome containing fraction (Ex) were separated on 12% SDS PAGE gels and transferred to PVDF membranes. Membranes were probed with anti-mouse HDGF or anti-actin as shown on the right side. Molecular mass marker lanes are indicated on the left. Signals corresponding to potentially N-terminal truncated HDGF are marked by asterisks.

Western blot analysis resulted in two signals at a position of approximately 37 kDa and 36 kDa in the exosome containing fraction collected from HepG2 cells transiently
Results

transfected with HDGF-C12A (figure 37, panel 1). Remarkably, the signal, which appeared at a slightly higher position, was resistant to the trypsin digest, whereas the second lower signal was sensitive so trypsin exposure. This indicated that the overexpression of HDGF C12A led to the presence of two subpopulations within the specimen, one which was within extracellular vesicles and the second one, which was located at the surface of vesicles. Furthermore, the analysis of supernatants collected upon each centrifugation step and the respective final exosome fraction (panel II, 1) revealed that the lower signal was nearly not detected in the supernatants but was enriched in the exosome containing fraction. Such enrichment could not be observed for the upper signal.

Regarding the result from the N-terminally truncated variants (figure 35) the respective protein was deleted in 11 or more N-terminal amino acid residues. The same result was obtained when exchanging both Cys residues C12 and C108 to Ala. However, the exchange Cys108 did not lead to the formation of a second smaller fraction, which was sensitive to trypsin exposure (figure 37, panel I.2). In the respective Western blots only a single signal was detectable.
4. Discussion

4.1 Alternative Splicing and functional consequences

Alternative splicing is a key mechanism for the generation of high diversity in the proteome. Recent studies estimated that the majority of the human genes undergo alternative splicing [133]. On the basis of this estimation the finding that human HDGF is alternatively spliced is not surprising. In accordance, alternative splicing events have been observed also in other members of the HRP family like LEDGF [11] and HRP-2 [165]. Moreover, in case of HRP-3 a putative alternative splicing variant can be found in the NCBI database (HRP-3: transcript X1 XM_006720554), even though this variant has not been verified experimentally.

In the following the expression of the novel HDGF isoforms and their potential functional roles are discussed.

4.1.1 Gene expression of alternative HDGF isoforms

The results of the present work revealed that alternative splicing of human HDGF mRNA generates at least two further isoforms, HDGF-B and HDGF-C. These two isoforms differ in the N-terminal part from HDGF-A. Interestingly, the novel isoforms HDGF-B and -C are not present in the genome of mice, although human HDGF-A and murine HDGF share a high similarity (86 %). In line with this observation are other studies, which demonstrated that alternative splicing events are relatively low conserved between the human and the mouse genome [189, 190].

Searching nucleotide databases for orthologous sequences revealed that the alternative exons for HDGF-B and -C can be found only in closely related species of the anthropoid lineage. This indicated that the event, which led to the existence of these alternative isoforms, occurred relatively late in evolutionary history.

Even though this is not issue of this work it should be mentioned that further predicted alternative HDGF transcripts in the NCBI database are available, indicating that there might be more than three different HDGF variants.

However, the presence of the mRNA is not immediately the verification that the respective sequence is translated into a protein and it is not that simple to distinguish biologically important variants from erroneous transcripts. The nature evolved several mechanisms which tightly control the subsequent translation process in order to prevent the expensive production of defective and potential harmful translation products [191]. One of the major surveillance mechanisms is the non-sense mediated mRNA decay (NMD) which eliminates transcripts with a premature stop codon [192]. A recent study,
working with EST-suggested alternative human isoforms, estimated that 35 % of these human EST-suggested isoforms are target for NMD surveillance process [193]. This NMD process does not concern one of the novel HDGF isoforms HDGF-B and HDGF-C, since they do not contain a premature Stop codon.

RNA expression of the two novel HDGF isoforms HDGF-B and HDGF-C could be verified (figure 8). The results of real-time qPCR analysis demonstrated that the previously described HDGF-A form is clearly the predominant form. HDGF-B and HDGF-C are expressed only at very low levels compared to HDGF-A (figure 9) in all investigated human cell lines under the tested conditions. This seems in line with the observations from Djebali et al. [142]. Their analysis of expression patterns revealed that cell lines tend to express several alternative transcripts at the same time but that in most cases one transcript is the dominating form. Such a dominant expression of one isoform seems to be the case also on protein levels [143]. Certainly, the result of the qPCR does not reveal whether cells express all variants at the same time or the majority of cells express solely the dominant HDGF-A form whereas a few cells of the studied population express one of the novel alternative isoforms at high levels.

Nevertheless, there might be specific conditions available, which lead to an upregulation of HDGF-B and/or HDGF-C and simultaneously in a decrease of HDGF-A expression levels. Such changes of alternative splicing patterns are observed for specific genes under different circumstances for example as a response to specific stimuli [194, 195] or as consequence of differentiation processes [196]. Also cancer related changes are known [197]. Regarding the role of HDGF-A in cancer development [31, 42, 62] these cancer-related changes are an interesting aspect. The identification of specific conditions which lead to an upregulation of the novel HDGF isoforms HDGF-B and -C would reveal more about physiological function and their biological relevance and is therefore an interesting and important project for the future.

### 4.1.2 Protein expression of alternative HDGF isoforms

HDGF-A exhibits an abnormal electrophoretic running behaviour in SDS PAGE [3]. A similar observation was obtained for HDGF-C and HDGF-B. Both migrated more slowly in SDS PAGE than was expected on basis of their predicted molecular weights. HDGF-C appeared at a molecular weight position of 37 kDa whereas HDGF-B was detected as a triple signal at around 37 - 40 kDa.

An interesting observation was that under non-reducing conditions a prominent signal was visible at a molecular weight position of about 90 kDa in case of HDGF-A. Similar signals were also detected for HDGF-B and HDGF-C but considerably weaker (figure 11). Thakar et al. provided evidence that mouse HDGF is able to form disulfide bridged
dimers via Cys108 [33]. The same Cys residues is available in human HDGF-A sequence (Cys108, figure 7) indicating that an identical mechanism is existent and most probably the reason for the observed 90 kDa signal in Western blot analysis. In the corresponding samples containing ectopically expressed HDGF-B and HDGF-C such a strong 90 kDa signal was not detectable. Apparently, HDGF isoforms have lost the ability to form such disulfide bridged dimers in a similar extent. This is surprising since the responsible Cys is present in both proteins (HDGF-B: Cys124; HDGF-C: Cys101) (figure 7). Possibly, the overall changes in HDGF-B and -C lead to an unfavourable position of the responsible Cys residues that reduces formation and/or stability of such disulfide bridged dimers.

The detection of the novel HDGF isoforms as endogenously expressed protein is challenging due to the limited sequence differences, the similar running behaviour and the low expression levels of HDGF-B and -C relative to HDGF-A (figure 9). Although the sequences of alternative isoforms have been present in the database for a while, only a limited number of studies designed their experiments in such a way, that HDGF-A was specifically addressed. One problem is that most commercially available HDGF antibodies will recognize all three HDGF isoforms. The similar electrophoretic running behaviour aggravates this problem. On the one hand the dominance of HDGF-A and the resulting strong signals in Western blot analysis may overwhelm weaker signals derived from HDGF-B or HDGF-C. On the other hand, situations in which HDGF-B and/or HDGF-C are indeed highly expressed in relation to HDGF-A, the respective signals may be accidentally assigned to HDGF-A due to similar positions in Western blot analysis. This underlines the necessity of isoform specific antibodies.

Interestingly, Nakamura et al. used initially an antibody which was directed against the outermost amino acid residues (SRSNRQKEYKC12) of HDGF-A. In immunofluorescence analysis using this specific antibody, a cytoplasmic staining was obtained in hepatoma HuH-7 cells [1]. This observation is in contrast to the majority of later studies which found that HDGF is mainly located in the nucleus of cells [17, 27, 77]. However, not much is known about the quality of the respective antibody, also because this specific antibody has not been used in later studies. Moreover, a novel antibody, directed against the same outermost amino acid residues of HDGF-A, was not functional in this present work (data not shown).

As consequence a second HDGF-A specific peptide antibody was produced, which was directed against another HDGF-A specific peptide (table 6, no 2). However, this antibody has a strong disadvantage in terms of specificity. It recognizes also other family members of the HRP-family. In contrast to the first HDGF-A specific antibody, this antibody was able to detect ectopically expressed HDGF-A (figure 11) and moreover did
not produce a signal in that samples which contained overexpressed HDGF-B and HDGF-C. Despite its ability to detect recombinant HDGF-A, this antibody failed completely in the recognition of endogenously expressed HDGF-A (figure 15, panel 1). Since qPCR revealed the dominance of HDGF-A, low endogenous protein levels are not the reason for this failure. Consequently, another explanatory model is required for this discrepancy. Endogenously expressed HDGF-A might undergo a posttranslational modification in the region that contains the epitope for the HDGF-A specific antibody (CGDLVFAKMGYPH25) and these changes might prevent antibody binding. This explanatory model requires that this modification does not occur or only partially in ectopically expressed HDGF-A. The high amounts of HDGF-A, that are obtained after transient overexpression, could overload the machinery responsible for the respective modification. Hence, a strong proportion of unmodified HDGF-A would be left, which can be still recognized by HDGF-A specific antibody.

In order to verify endogenous protein expression of HDGF-B and HDGF-C, peptide specific antibodies were used. Interestingly, HDGF-B specific antibody recognized only N-terminally tagged HDGF-B and failed to detect C-terminally tagged HDGF-B (figure 13 & 14). This led to the assumption that recombinant HDGF-B was N-terminally truncated while this process did not occur or only partly in case of N-terminally tagged HDGF-B. This truncation process might be artificially induced by its overexpression in cells. However, if truncation occurs also in the case of endogenously expressed HDGF-B, the detection will be anyway problematic due to the potential loss of the HDGF-B specific epitope.

Unfortunately, the HDGF-B specific peptide antibody failed to precipitate and detect HDGF proteins in cell lysates. Instead, the antibody precipitated and detected proteins in the respective precipitate with an electrophoretic mobility in the range of 70 to 100 kDa that were not detected by the goat anti pan-HDGF antibody (figure 15, panel 2 & 4). This strongly indicates that this antibody recognizes other proteins than HDGF. Due to this the antibody was firstly excluded from further studies. On the basis of this data, it cannot be answered whether HDGF-B protein is expressed.

Endogenous protein expression was confirmed for HDGF-C by the use of HDGF-C specific peptide antibodies. In immunoprecipitates HDGF-C was detected (figure 15, panel 3), indicating that HDGF-C is expressed in cells. The examination of human tissue samples (figure 16) provides a further hint for the presence of HDGF-C. Interestingly, detected signals were detected at a slight higher molecular weight position as observed for ectopic overexpression and immunoprecipitation. Posttranslational modifications could be a reason.
4.1.3 Structural differences in HDGF isoforms

HDGF-A consists of the structured N-terminal hath domain (1-100 amino acid residues) and the not well defined C-terminal part (101-240 amino acid residues) (figure 2) [13]. The alignment of the sequences revealed that changes in HDGF-B and HDGF-C affect the first two β-strands, which are part of the anti-parallel β barrel (figure 7). Such splicing events that affect rather structured and well-conserved regions of a protein than non-structured and more variable parts are not that rare as might be expected beforehand. Indeed, a study from Birzele et al. demonstrated that from the studied set of data approximately only half of the splice events affect variable protein regions, whereas the other half concerned well defined structures of proteins [198]. However, screening the literature did not lead to the discovery of further alternative isoforms within the PWWP protein family with alterations in the PWWP domain.

In silico models for the first N-terminal 116 and 93 amino acids of HDGF-B and HDGF-C, calculated by Dr. Waespy for our publication, demonstrated that the splicing events resulted in drastic structural differences [132]. In HDGF-B the first two β-strands can be formed by amino acid residues of the new alternative N-terminal part, so that the characteristic β-barrel still appears in the model with an extended loop between the β-strands β1 and β2. Strikingly, the extended loop consists of an additional α-helix. By contrast, in the model of HDGF-C β1 and β2 strands are completely lost and as consequence the characteristic β-barrel cannot be formed at all.

A further distinguishing feature of HDGF-B and -C was the loss of the PHWP motif, that constitutes the N-terminal part of the second β-strand in HDGF-A (Pro24-His25-Trp26-Pro27 in HDGF-A) (figure 7). At the respective position in HDGF-B (Arg40-Ala41-Thr42-Pro43) only the Pro residue at the fourth position (Pro43) can be found which corresponds to the Pro 27 in HDGF-A. In HDGF-C no obvious similarities to the PHWP are available. In the view of the fact that the PHWP motif contributes to the overall stability of the PWWP domain by forming hydrogen bonds with adjacent amino acid residues in the β-strands as well as by supporting a specific loop conformation [199], the loss of the PHWP motif in HDGF-B and HDGF-C is even more intriguing.

4.1.4 Functions and binding properties of the novel HDGF isoforms

Several groups used siRNA to silence HDGF expression. Only in some cases they used sequences which were directed against the N-terminal part, so that they specifically silenced only HDGF-A and not the two remaining isoforms [64, 153]. However, most groups utilized siRNA for their experiments which are able to silence all three isoforms [40, 63]. In general all these studies showed that reduction of HDGF expression results
in reduced proliferation, migration and invasion behaviour of cells. Consequently, it is not possible to conclude a specific function for one of the novel isoforms from these studies.

4.1.4.1 Proliferation

Growth stimulating activity belongs to one of the most studied effects of HDGF-A [1, 2, 17, 28, 50]. However, how HDGF-A exerts exactly its proliferative effect has not been clarified yet. Two independent pathways were suggested [41]. The first pathway requires nuclear localisation and the C-terminal non-hath region of HDGF-A [17]. The second pathway depends on the amino acid residues 81-100 in HDGF-A which provide a putative binding site for a yet unknown extracellular plasma membrane receptor [41]. Recently, surface nucleolin was discussed as potential receptor of HDGF [42].

On basis of theoretical considerations HDGF-B and –C should have the ability to promote cell growth, because they fulfil the mentioned criteria. Both contain the two NLS sequences and can be found in the nucleus upon transient overexpression (figure 12). In addition, both have the full non-hath region as well as the putative receptor binding site (figure 7). Therefore, both putative pathways are reasonable.

Indeed, like already observed for HDGF-A overexpression of HDGF-B stimulated actually the growth of Cos7 cells (figure 17). Surprisingly, such an effect could not be determined for HDGF-C. In the light of the study of Kishima et al., who demonstrated that overexpression of HDGF specific non-hath part alone is able to stimulate proliferation [17], this is surprising because the complete non-hath region is present in HDGF-C. Hence, the N-terminal part of HDGF-C seems to interfere in some way.

Whether the isoform HDGF-B and HDGF-C are able to utilize the putative receptor mediated pathway, cannot be answered from our data. Treatment with recombinant protein did not result in a significant enhanced proliferation, neither for HDGF-B and HDGF-C nor for HDGF-A (data not shown). Since different independent studies demonstrated that exogenous supplied HDGF-A stimulates cell growth, either the recombinant protein was not functional or the selected conditions of the assay were not appropriate. Nevertheless, the results of the secretion behaviour revealed that HDGF-B and HDGF-C are not present as free protein in the extracellular space but on the surface of extracellular vesicle (figure 31). Thus, the surface localisation would theoretically allow that HDGF-B and HDGF-C interact with the receptor protein. However, respective experiments with exosome collected from overexpressing cells did not show any proliferative effect on cells (data not shown). But also here it remains questionable whether appropriate conditions were selected.
4.1.4.2 Chromatin association

During this work the interaction to chromatin was studied, which is an established interaction of HDGF-A [49] as well as of HDGF related proteins LEDGF [160] and HRP-2 [49]. Chromatin association depends on the PWWP domain [16], therefore the altered N-termini of HDGF-B and -C might change interaction to this complex structure in some way. To address this question a chromatin binding assay was performed. Chromatin binding can be complex and depends on protein-DNA interaction and/or on protein-protein interaction. However, the performed assay cannot reveal how the protein of interest associates with this complex molecule.

Like HDGF-A, HDGF-C was identified in the chromatin associated fractions (figure 18). Surprisingly, HDGF-B was not able to interact with chromatin, demonstrating that this protein cannot fulfil chromatin-related functions of HDGF-A. The Western blot analysis of overexpressed HDGF-B indicated that this protein loses its N-terminal part. Whether the overall changes or the loss of the N-terminal part is the reason cannot be answered.

The PWWP domain of HDGF has been reported to contain DNA binding ability due to its characteristic positive patches on the surface [16]. Yang et al. demonstrated that HDGF-A binds to a specific DNA sequence and by that mediates transcription repression with subsequent consequences in differentiation processes [25, 200]. Until now, this is the only function of HDGF which is directly linked to its chromatin association.

The same study revealed that binding to the specific DNA element requires the full length PWWP domain. Variants, which were truncated in the first 30 N-terminal amino acid residues bound to the DNA element only weakly. Hence, due to the missing N-terminal part also HDGF-C might not be able to bind this specific DNA sequence.

Other studies suggested that the PWWP domain facilitates DNA binding in a more unspecific manner [16, 158, 201]. Lukasik et al. suggested that the positive surface of the PWWP domain, constituted by the positively charged Lys residues (position 8, 19, 21, 61, 72, 75, and 80 in HDGF-A) and Arg79 mediate binding to the negatively charged DNA molecule. Interestingly, HDGF-C as well as HDGF-B lacks of amino acids corresponding to lysine residues Lys8, Lys19 and Lys21 (figure 7).

Furthermore, PWWP domain mediates binding to another chromatin component. The HRP family members HRP-2 [202] and LEDGF [203] as well as other PWWP domain containing proteins such as bromo and plant homeodomain finger-containing protein 1 (BRPF1) are able to bind to lysine methylated histones [202]. Although this interaction to methylated histones was not exactly shown for HDGF-A, the high similarity of the corresponding PWWP domains, especially in the HRP family (figure 1), suggests that a similar interaction can be expected for HDGF-A, too. Along this line Zhao et al. identified several histone proteins in the interactome of HDGF-A [5].
All histone binding proteins of the PWWP protein family contain a conserved aromatic cage, constructed by three aromatic residues, which allows the specific recognition of methylated lysine residues of histones. This aromatic cage (in HDGF-A constituted by following residues Tyr23, Trp26 and Phe44) is not available in HDGF-B and HDGF-C due to the missing Tyr23 and Trp26, indicating that such an interaction to methylated histones is not likely.

Furthermore, interaction to other chromatin associated proteins might mediate the chromatin association. Zhao et al. identified several other chromatin associated proteins in the interactome of HDGF-A [58] which may also interact with HDGF-C but with respect to the present results not with HDGF-B.

Interestingly, chromatin binding seems not to correlate with proliferation activity of HDGF isoforms. Although HDGF-B could not associate with chromatin, it was still able to stimulate proliferation, indicating that proliferation is independent from chromatin interaction. The reversed effect was observed for HDGF-C. Although it is able to associate with chromatin, it does not enhance cell proliferation. This is in line with the observations that the non-hath region of HDGF, which does not participate in chromatin association [13, 16, 25], is able to enhance proliferation [17].

### 4.1.4.3 Dimerisation of HDGF isoforms

HDGF-A is able to dimerise via an unusual domain swap mechanism, mediated by the PWWP domain [26]. According to Sue et al. [26], the dimerisation mechanism depends on the N-terminal β1 and β2 strands, which are exchanged between the two HDGF-A monomers. The long flexible loop, localised behind the second β strand, serves thereby as hinge loop and connects both monomers with each other.

Considering that the N-terminal part including the first two β-strands, is altered in HDGF-B and -C, it seems reasonable to suppose that dimerisation behaviour is changed, too. According to the in silico model, HDGF-B contains the required structural elements, since the altered N-terminus consists of two β-strands and also the flexible loop is available [204]. Thus, theoretically dimerisation can be possible. However, the loop, placed between β1 and β2, is extended compared to HDGF-A and might influence the dimer formation in some way. By contrast, the in silico model of HDGF-C revealed that HDGF-C lost both N-terminal β-strands nearly completely [204], making the domain swap mediated dimerisation rather unlikely.

In the view of this theoretical considerations the result of BiFC assay (figure 19) is surprising because not only HDGF-A and HDGF-B but also HDGF-C have been found to form HDGF dimers. Moreover, according to the results of this assay all possible combinations of HDGF isoforms appeared to be feasible.
As mentioned before, dimerisation can also depend on disulfide bridges. Thakar et al. demonstrated that murine HDGF dimerise via Cys108 [33]. Indeed, after overexpression of HDGF isoforms signals at about 90 kDa, especially for HDGF-A, were obtained in Western blot analysis under non-reducing conditions, which disappeared under reducing conditions (figure 11).

However, although BiFC assay is an established way to study direct interaction processes in the living cell, a further explanation for the observed BiFC signal cannot be excluded. It is possible that a third mutual interaction partner brings the HDGF molecules in such a close proximity, that complementation of the respective linked EYFP fragments arises by this way and not by a direct interaction.

The functional consequence of homo- and heterodimerisation can be only speculated. Homodimerisation of HDGF-A enhances binding affinity to heparin probably by the formation of a contiguous and consequently increased binding site [26]. Since binding to DNA depends on the same binding site [16], homodimerisation of HDGF-A might also increase the affinity to DNA. Whether putative heterodimerisation of HDGF-A with one of the novel isoforms leads to a similar effect is unclear. However, as described before, some of the positively charged amino acid residues of the heparin/DNA binding site in HDGF-A are not available in HDGF-B and HDGF-C. Therefore, such an enhancement of the binding affinity to the same extent is doubtful.

Heterodimerisation of isoforms, deriving from the same gene, can be observed for several splice variants such as for isoforms of the inderminate domain (IDD) 14 transcription factor [205] or the prolactin receptor (PRLR) [206]. In both cases the heterodimerisation has a regulative consequence [205, 206]. In case of IDD14 transcription factor the alternative isoform, which lacks a functional domain, attenuates the activity of the IDD14 transcription factor by binding to the active factor [205]. Such a model would be interesting, in particular when regarding the pathophysiological role of HDGF in cancer and tumour development. Besides, heterodimerisation might combine different functions or interaction partners. Regarding that HDGF-B and -C exhibited quite different interaction behaviour; this opens up entirely new possibilities.

**4.1.4.4 Interaction patterns of HDGF isoforms**

HDGF-A has been described as multifunctional protein, which participates in different cellular processes. This multifunctional role is also mirrored by the identification of diverse HDGF-A interaction partners [5, 7]. Zhao et al. discovered in their multi-interactome study that most protein-protein interactions depend on the N-terminal hath region of HDGF-A. From the 106 identified proteins only 5 proteins were detected in co-precipitations of the C-terminal non-hath region, displaying the importance of the hath
region in the majority of HDGF-A-protein interactions [5]. In line with this observation are the results of the present co-precipitations within this study (figure 20). The N-terminally altered isoforms were not able to interact with some of the known HDGF-A interaction partners and beyond that they co-precipitated other proteins, which in turn were not present in the co-precipitates of HDGF-A. However, the alteration in HDGF-B and HDGF-C were negligible for interactions to Hsc-70 and ADP/ATP Translocase 2, because these proteins were identified in all three co-precipitates of HDGF-A, -B and -C (figure 20).

The interactome of HDGF-A consists of multiple RNA binding proteins. This discovery led to the assumption that HDGF-A is involved in different RNA related processes such as RNA splicing or translation [5, 7]. Besides, the identification of numerous ribosomal proteins in the interactome suggested that HDGF-A is a putative candidate involved in ribosome biogenesis [5]. In line with this is the detection of a further ribosomal protein (60S ribosomal protein L6) in the co-precipitate of HDGF-A (figure 20). Interestingly, this ribosomal component was not observed in the corresponding eluates of HDGF-B and HDGF-C. However, the present data is not nearly sufficient to allow a complete exclusion of an interaction of HDGF-B and -C with this specific ribosomal protein as well as with other ribosomal components. Subsequent Western blot analysis with corresponding antibodies would clarify the question whether these isoforms are able to interact with some ribosomal components.

Another RNA binding protein found in the HDGF-A co-precipitate was nucleolin [5, 123] (figure 20). Nucleolin plays among other RNA related functions also a role in ribosome biogenesis [207]. Interestingly, nucleolin has been reported to function as surface receptor [208, 209] and moreover HDGF-A is able to interact with surface nucleolin [42]. Strikingly, in contrast to HDGF-A, the novel isoforms HDGF-B and HDGF-C lost the ability to co-precipitate nucleolin. Furthermore, the first 15 amino acid residues of HDGF-A are essential for its capability to co-precipitate nucleolin and their missing in HDGF-B and -C is probably the reason why nucleolin was not detected in the corresponding co-precipitates. This became evident by the analysis of co-precipitates obtained with the ΔN15 HDGF-A variant. This construct was deleted in the first 15 N-terminal amino acid residues. Respective Western blot analysis of its eluate exhibited substantially decreased levels of co-precipitated nucleolin (figure 22).

Although the presence of the first 15 N-terminal amino acids in HDGF-A are essential for its nucleolin interaction (figure 22), a respective construct containing solely these first 15 or 30 amino acid residues added to SNAP-Strep sequence, was not able to co-precipitate nucleolin (figure 22). In line with this are the observations from Zhao et al. [5]. They identified nucleolin in the eluate obtained from the HDGF-A full length protein, but
not in the precipitates obtained from the HDGF-A *hath* region. This suggests that the complete protein is involved in the interaction.

Apart from nucleolin also YB-1 was detected in HDGF-A co-precipitates, while it was absent in co-precipitates of HDGF-B, -C and the NΔ15 HDGF-A variant. Again the first 15 amino acid residues of HDGF-A appeared to be critical for the co-precipitation, an obvious similarity to the co-precipitation of nucleolin. Similar to nucleolin YB-1 is a RNA and DNA binding protein thought to be involved in multiple RNA and DNA related processes [170]. As already shown for nucleolin [123], the interaction of HDGF-A with YB-1 relies on the presence of RNA. Pre-treatment with RNase abolished the co-precipitations of these proteins (figure 23).

Whether HDGF-A has the ability to bind to RNAs directly has not been resolved until now albeit Zhao et al. [5] discovered some rRNAs in HDGF-A precipitates. Interestingly, like observed for the majority of protein interactions, the non-*hath* region of HDGF-A was dispensable for the co-precipitation of these rRNAs. It is conceivable that a putative RNA binding potential of HDGF-A *hath* region relies on the same binding site as suggested for DNA binding [16]. Correspondingly, as discussed beforehand (section 4.1.4.2), the binding potential might be reduced in HDGF-B and HDGF-C due to the loss of some of those positively charged amino acid residues that comprise the DNA and with that also the potential RNA binding site in the *hath* region [16]. The lost or reduced RNA binding potential might be also the reason why HDGF-B and -C as well as the constructs containing the first 15 or 30 N-terminal amino acid residues of HDGF-A failed to co-precipitate nucleolin and YB-1. However, the results of Zhao et al. [5] implicate that at least in the case of nucleolin the C-terminal non-*hath* region of HDGF-A seems to be involved in the interaction in some way.

Interestingly, Chen et al. were able to co-precipitate nucleolin and YB-1 with each other from Jurkat cytoplasmic extracts and also this interaction between these two proteins was abolished by RNase pre-treatment. They discovered that YB-1 and nucleolin are required for the stabilisation of interleukin-2 (IL-2) mRNA by the formation of a mRNP complex [210]. Both proteins are also present in other mRNP complexes and both influence stability and mRNA turnover [211-213]. For instance, nucleolin was described as B-cell lymphoma 2 (bcl-2) stabilizing factor [214]. As a matter of fact HDGF-A is a component of this complex [123] but its functional role in this complex has remained mostly unknown.

Whether HDGF-A forms a mutual RNP complex with nucleolin and YB-1 or individual complexes with each protein cannot be answered from this set of data but further clarification would be interesting, since this may enlighten also the functional role of HDGF-A in such RNP complexes.
In addition to its function in these RNP complexes, HDGF-A interacts with surface nucleolin [42]. Since HDGF-B and -C are not able to interact nucleolin, it is unlikely that they are able to induce downstream signalling pathway via this HDGF/nucleolin axis. Strikingly, while HDGF-B and -C were not able to interact neither with YB-1 nor with nucleolin, they co-precipitated proteins of the cytoskeleton, which in turn were not found in the co-precipitate of HDGF-A. Among these cytoskeleton proteins tubulin was identified. The observed co-precipitation of tubulin and HDGF-C coincided with the immunofluorescence analysis. HDGF-C appeared in granule-like structures, which clearly co-localised with the microtubulin network (figure 24). The absence of tubulin in the HDGF-A is in agreement with other interactome studies [5, 7], since these studies did not identified tubulin as an interaction partner of human HDGF-A either. Furthermore, also in the interaction database Biological General Repository for Interaction Datasets (BioGRID) (http://thebiogrid.org) [215] tubulin was not among the 213 entries for human HDGF-A interaction partners. Interestingly, tubulin interaction is also known for another HRP family protein [216]. HRP-3, whose expression is restricted to neurons and glial cells [3, 9], binds directly to tubulin and by that stabilises microtubules and facilitates their proliferation. Its tubulin promoting role was linked to its positive effects on neurite outgrowth. Intriguingly, the binding to tubulin is mediated by the hath domain of HRP-3 while the non-hath domain is again dispensable for the binding [216]. In the view of the fact that the hath domains of HDGF-A and HRP-3 share such a high homology (figure 1), it is quite surprising that HDGF-A failed to bind to tubulin. This is even more intriguing regarding that HDGF-B and HDGF-C (figure 20) and furthermore also the NΔ15 HDGF-A Strep variant were able to co-precipitate tubulin (figure 22). Of course, whether direct binding to tubulin is involved as observed for HRP-3 [216] or indirect binding cannot be decided on the basis of the present data. But due to the similarity to HRP-3 a direct interaction is likely and in this line HDGF-B and HDGF-C might be two further candidates, which trigger microtubule assembly and stability. Taken together, the results of the precipitation experiments indicate that the first N-terminal amino acid residues of HDGF-A prevent the interaction to tubulin in some way. However, a simple explanation how tubulin binding occurs and beyond that how binding is disturbed by the outermost N-terminal peptide of HDGF-A cannot be provided. The overall defined structure of the hath domain does not seem to be required for tubulin interaction since HDGF-C, which undergoes drastic structural changes in the hath region, is still able to interact with tubulin. Tubulin-binding motifs are commonly characterised by the presence of positively charged amino acids which mediate binding to the C-terminal acidic part of tubulin [217-219]. Indeed, in the C-terminal part of the
hath region several positively charged amino acid residues are available in all three HDGF isoforms (in HDGF-A Lys70, Lys72, Lys75, Lys78, Arg79, Lys80) (figure 7). These residues are also present in HRP-3 (in HRP-3 Lys69, Lys71, Lys74, Lys77, Arg78, Lys79) (figure 1). Actually, El-Tahir et al. provided first valuable hints that the mentioned region is involved in tubulin binding. The mutation of the positively charged Lys and Arg residues (Lys74, Lys77, Arg78 and Lys79) to an uncharged Asn in HRP-3 reduced the stabilisation activity on microtubules significantly [216]. However, whether these amino acid residues participate in tubulin binding needs to be addressed in future studies.

Intriguingly, the HDGF-A interaction partner YB-1 is also a tubulin-binding protein and like HRP-3 it promotes microtubule assembly [220]. The release of YB-1 from inactive mRNPs, relieves transcriptional repression of the respective mRNA [221]. Chernov et al. proposed that YB-1 dissociation from mRNPs occurs through tubulin binding [220]. Interestingly, posttranslational phosphorylation decreases affinity of YB-1 to RNAs and might be a tool for its regulation [221]. Regarding that first 15 aa of HDGF-A are essential for YB-1 co-precipitation and at the same time the presence of this peptide in HDGF-A prevents its tubulin binding, a comparable mechanism is conceivable for HDGF-A. Thakar et al. proposed that dephosphorylation of Ser165 results in N-terminal truncation [33]. Hence HDGF-A release from mRNPs and the subsequent binding to tubulin might be induced by posttranslational dephosphorylation at Ser165 and subsequent N-terminal truncation.

A further interesting result was the detection of dynein, a microtubuli minus end directed motor protein [172], in the co-precipitates of HDGF-B and -C (figure 20). This finding leads to the assumption that HDGF-B and HDGF-C might be involved in transport related processes either as cargo or as part of the transport machinery, for example as cargo linker. While dynein was clearly present in co-precipitates, its antagonist kinesin, a motor protein which is directed to the plus end of microtubulis [172], was absent. Consequently, the involvement in transport refers rather to processes from the cell periphery towards the cell centre. In case of HDGF-C this could be further confirmed by the immunofluorescence analysis (figure 24). Strong co-localisation was observed for HDGF-C and dynein. Due to the lack of a functional HDGF-B specific antibody, similar immunofluorescence analysis could not be performed.

Surprisingly, HDGF-C appeared to interact directly or indirectly also with other elements of the cytoskeleton. Actin and vimentin were identified in the co-precipitates of this isoform (figure 20). Regarding that vimentin, tubulin and actin belong to distinct elements of the cytoskeleton, this was unexpected. Nevertheless, linkages between the cytoskeleton elements are well known and several proteins have the ability to interact with the different cytoskeletal proteins and thereby serve as a linker between these
elements [222]. This linker protein interacts directly as reported for CacyBP/SIP (calcyclin (S100A6) binding protein and Siah-1 interacting protein), that binds actin as well as tubulin directly [223]. In other cases interactions are more indirect and mediated by auxiliary binding partners [224]. A further link is given by the observation that the motorproteins dynein and kinesin are responsible for the transport and distribution of vimentin within the cell, a process required for the organisation and maintenance of vimentin networks [225, 226]. Moreover, also direct interaction between actin and vimentin has been reported [227].

The discovery of these cytoskeletal interaction partners opens a wide field of possibilities about the functional role of HDGF-C and also HDGF-B. The co-precipitation of different interaction partners conveys the impression that their role is quite distinct from that of the main HDGF-A form. HDGF-C might constitute a further direct or indirect linker protein between the cytoskeleton elements; it might participate in retrograde vimentin transport or in other transport related processes. Like suggested for HRP-3 [216], HDGF-B and -C might be also involved in cytoskeleton dynamics through the stabilisation and promotion of microtubule assembly. To clarify the exact role further studies are required. However, overlapping roles or functions cannot be excluded, on the one hand due to the presence of common interaction partners such as Hsc70 (figure 20) and on the other hand due to the finding that heterodimerisation of the isoforms (figure 19) seems to be possible. Interestingly, the different interaction partners of HDGF-A, -B and -C can be found in Staufen-2 protein complexes. Staufen-2 proteins bind directly to Hsc-70 and are associated to RNA binding protein YB-1 through RNAs. Moreover, the cytoskeletal proteins tubulin and actin were identified in corresponding precipitates [228]. Several studies suggested that this complexes are transported in kinesin and dynein dependent mRNP transport along microtubules [228-230]. However, Stau2 protein is mainly expressed in brain and only expressed at low levels in other tissues [229], thus in the studied cell systems (HepG2 cells and dermal fibroblast) it does not seem to be relevant. Nevertheless, since all the different interaction partners are involved in mRNP transport, this mechanism is interesting for future studies.

### 4.1.5 HDGF and Secretion

Numerous studies demonstrated that exogenous supplied HDGF-A stimulates migration [66] and proliferation [1, 2, 37, 38, 50], indicating that HDGF-A realizes in addition to its intracellular functions also extracellular functions. This is consistent with other studies demonstrating that HDGF-A can be found in the surrounding media from different cells like neurons [77], glioblastoma [55], and muscle cells [78] or in serum collected from lung cancer patients [80]. Nevertheless, until now not much is known about its cellular
trafficking, how exactly HDGF-A exits the cell and about possible underlying regulatory processes.

Some studies discussed that the extracellular presence of HDGF is more a consequence of passive release [44, 77]. Indeed, cells that undergo necrosis but not apoptosis release HDGF passively [77]. However, passive release does not rule out the possibility of active secretion. For instance, HMGB1, which shares 35 % similarity with HDGF-A [1], can leave the cell by both active secretion and passive release [81, 231, 232]. HDGF-A does not contain a N-terminal hydrophobic signal sequence required for the ER/Golgi mediated pathway (table 8; appendix A1) and simultaneously, the changes through alternative splicing in the N-terminal part of HDGF-B and HDGF-C does not create a novel secretion peptide either (table 8; appendix A2-A3). As consequence putative active secretion of all these HDGF isoforms has to occur via alternative secretion pathways.

As described in the previous chapters, alternative splicing of HDGF leads to the generation of isoforms with key changes in their overall structure, their function as well as in their interaction behaviour. It appears obvious that also secretion of HDGF-B and HDGF-C is altered, especially since the outermost N-terminal amino acids of mouse HDGF are critical in its secretion [33]. In contrast to the full length mouse HDGF, truncated HDGF, deleted in 11 or more N-terminal amino acid residues, could not be found in extracellular media after 24 h incubation. The same was found for mouse HDGF S165A, a variant which is N-terminal truncated, too [33]. Indeed, a comparison of human HDGF release into the conditioned media over the course of time (figure 25) revealed that HDGF-B like HDGF-A S165A was below the detection limit in the extracellular media. In addition, extracellular levels of HDGF-C were considerably lower compared to HDGF-A. According to this result HDGF-A is the main secreted HDGF variant and, as discussed by Thakar et al. [33], its secretion can be controlled by absence/presence of the outermost N-terminal amino acids.

The fact that alternative spliced variants exhibit different secretion behaviours was also described for other growth factors such as transforming growth factor beta (TGF β) [233] or vascular endothelial growth factor (VEGF) [234]. On the condition that expression levels are comparable, the release of different protein levels over the course of time hints towards different underlying regulatory processes and/or alternative secretion processes.

4.1.5.1 HDGF and the presence in extracellular vesicles

As mentioned, none of the HDGF isoforms contains a classical secretion signal, thus putative active secretion has to occur via an alternative pathway. In line with this was the observation that the inhibitor Mon [178], which interferes with the ER/Golgi mediated
secretion pathway, did not inhibit HDGF-A release (figure 26, panel 1). Instead, HDGF-A secretion was inhibited specifically by treating the cells with DMA. Along this line, the release of ectopically expressed HDGF-C (figure 26, panel 3) and of endogenous HDGF (figure 26, panel 4) from HeLa cells decreased after DMA treatment, too. The observed decline, induced by DMA, was not as strong as observed for ectopically expressed HDGF-A. However, since longer incubation times were required (up to 42h) to be able to detect HDGF-C and endogenous HDGF in the conditioned media, the direct comparison of the observed DMA effects is certainly difficult. For instance, cells might become accustomed with the course of time to the changed conditions and are able to compensate effects. However, literature addressing such specific long term effects of DMA on secretion is not available.

The identification of a specific inhibitor supports the concept of an active secretion mechanism indirectly. DMA is a known inhibitor of exosome release [177], decreasing the exosome mediated release of several proteins such as Hsp90 [235], Hsp60 [236] or the receptor protein sortilin [237]. Hence, it is an obvious possibility that HDGF-A and -C leave the cell via exosomes. This assumption is consistent with the recent proteomic studies which identified HDGF in exosomes [87, 89, 94].

Savina et al. [177] assumed that the decrease of exosome release caused by DMA, depends on a decline in intracellular Ca^{2+} levels. However, the observation of the inhibitor assay does not exclude other alternative secretion pathways. An elevation of intracellular Ca^{2+} levels was reported to induce fusion of lysosomes with the plasma membrane [238]. Furthermore, microvesicle release through shedding from the plasma membrane is also stimulated by an increase of intracellular Ca^{2+} levels [239]. Thus, changes in intracellular Ca^{2+} levels might control also other pathways than the exosome mediated pathway. But respective literature concerning such DMA effects on other alternative secretory pathways was not found.

Nevertheless, the analysis of exosome enriched samples led to the same conclusion (figure 27 & 28). Endogenously expressed HDGF as well as all ectopically expressed HDGF isoforms and HDGF-A S165A were detectable in exosome enriched fractions (figure 27 & 28).

As mentioned earlier, in the present study differential centrifugation was performed to isolate exosomes from the conditioned media. It is commonly known that smaller amounts of shedding microvesicles and apoptotic blebs of equal size may co-sediment with the exosomes. Therefore, obtained fractions might contain subpopulations of other extracellular vesicles, which potentially can be the source for the detected HDGF variants [99, 240].
However, Xu et al. used an elaborate purification method and could demonstrate that HDGF is also present in these highly purified exosome fraction [89], strongly suggesting that HDGF can use indeed this vehicle to exit the cell. Which HDGF isoform was identified by Xu et al. cannot be answered on basis of their published data. But the general dominance of HDGF-A in all investigated cell lines (figure 9) implicates that they detected HDGF-A.

Most strikingly, even though HDGF-B and HDGF-A S165A were below detection limits in the conditioned media (figure 25), they were identified highly enriched in the exosome fractions (figure 27). The same kind of enrichment was observed for exosomal marker protein CD63 (figure 27), a protein which is commonly known to be highly enriched in exosomes [107, 241]. In contrast, HDGF-A, HDGF-C (figure 25) as well as endogenous HDGF from some of the studied cell lines (HeLa, HaCat, MCF-7) (figure 28) were not only detectable in those supernatants (SN1-SN4), collected before the actual exosome sedimentation step, but also in the SN5, collected after exosome sedimentation. In all cases these respective proteins were not enriched in the final exosome containing fraction to the same extent like CD63. This obvious difference to the exosomal marker CD63 leads to the assumption that these proteins exist additionally as free protein in the extracellular space.

Indeed, subfractions of HDGF-A and endogenous HDGF were still detectable in the exosome depleted supernatants (SN5*), obtained after an additional ultracentrifugation for 24 h (figure 30). Therefore, these subfractions are not vesicle associated and present as free protein in the media. Like the exosomal marker CD63, the other HDGF variants (HDGF-B, HDGF-A S165A and also HDGF-C) were not detectable in SN5*, indicating that these proteins are only present in the vesicle associated fraction.

Of note, another explanation for the detection of HDGF-A in exosome depleted supernatants could be that the protein derived from defect vesicles. But since HDGF-B and HDGF-A S165A were not detected in the vesicle depleted supernatants under the same conditions, this is unlikely.

The presence in the extracellular medium as free protein or within vesicles can have different consequences in terms of function and half life. While proteins located within extracellular vesicles are protected by the vesicular membrane, free proteins might have shorter half times in the extracellular medium. Furthermore, functional roles might be different. Previous studies have not directly examined whether HDGF-A is present as free protein or within vesicles but numerous studies demonstrated that extracellular supplied HDGF-A induces cell growth and migration [1, 38, 46, 50, 66]. Indeed, several studies suggested that HDGF-A is able to exert its proliferative effect via until now
unknown receptor(s) present on the plasma membrane [41, 79]. Intra-vesicular HDGF-A would not be able to interact with surface receptors. Furthermore, cells are able to internalise free HDGF-A [167]. Nonetheless, the detection of additional free HDGF-A and free endogenous HDGF raises the question which further alternative pathway, apart from an extracellular vesicle mediated pathway, is used by these proteins to leave the cell and end up as free protein in the extracellular space.

It has been reported that multiple secretion mechanisms can be involved in the release of the same protein like for the cytokine interleukin-1beta (IL-1β). IL-1β can leave the cell via different types of vesicles including exosomes [242] and shedding microvesicles [243] derived from the plasma membrane. Moreover, it is able to exit the cell via secretory lysosomes [244, 245]. HMGB1, a protein which shares 35 % similarity with HDGF-A [1], was identified in extracellular vesicles [246]. In the same study it was shown, that HMGB1 was still detectable in exosome-depleted supernatants, likewise indicating that more than one secretion process might be involved at the same time. This finding was supported by another study reporting that HMGB1 leaves the cell via secretory lysosomes [232].

### 4.1.5.1.1 Endogenous HDGF in extracellular vesicles

Consistent with the recent proteomic studies [87, 89, 94], endogenous HDGF was detected in exosome fractions enriched from different cell types. As discussed in the beginning, with the discovery of the novel HDGF isoforms, there remains always an uncertainty, which isoform is detected when using an anti pan-HDGF antibody. This is strengthened by the discovery that all HDGF isoforms appeared to be released via extracellular vesicles. Thus, the detected signal could derive from the presence of all three isoforms. But, due to the great dominance of HDGF-A in all studied cell types (figure 9) it is more than likely, that HDGF-A will be found in these cells as well as in their surroundings.

An interesting finding was that endogenous HDGF was not detected in all exosome fractions. Exosome enriched fractions collected repetitive times from HepG2 cells did not contain HDGF in detectable amounts. However, overexpressed HDGF variants in these cells can be released by these cells (figure 25 & 28). In extracellular vesicles collected from MDA-MB-231 cells HDGF signals appeared only weak in Western blot analysis (figure 29). But according to literature HDGF could be isolated from the conditioned medium of MDA-MB-231 cells after purification with heparin binding columns [247], indicating that HDGF is also secreted from those cells but probably in lower amounts so that it remained almost below the detection limit. The simultaneous analysis of the
cleared lysates demonstrated that different cell types expressed HDGF at comparable levels (figure 29). Therefore, cell specific HDGF abundance in exosome fractions seemed to be rather caused by different selective protein loading into extracellular vesicles and was not a consequence of different expression levels. Along this line Jeppesen et al. demonstrated that HDGF belonged to that group of proteins, which were more abundant in exosomes derived from metastatic cells compared to those derived from non-metastatic cells. These observed changes in exosomes did not correlate with changes on mRNA levels. However, since they did not compare corresponding protein expression levels, observed changes in protein abundance as consequence of altered protein levels within the cell cannot be excluded completely [87]. This is even more important in the light of those studies, which correlated elevated HDGF protein levels with metastasis and cancer progression [32, 61, 248, 249].

The molecular protein composition of exosomes is not just a simple reflection of protein composition in cells. Exosomes are enriched in specific proteins, whereas others are lower concentrated or completely absent [105, 183]. Therefore, protein sorting into exosomes is regarded as specific process, which is regulated. Underlying sorting processes are far from being understood [112].

In this context the cell type specific appearance of two HDGF polypeptides in exosome enriched fractions at molecular weight positions of 37 and 39 kDa is interesting (figure 29). The 39 kDa HDGF signal is the predominant form in HaCat derived extracellular vesicles while this variant is underrepresented in the total cell lysate and hardly detectable. This clearly shows that this 39 kDa HDGF form is highly enriched in exosome fractions and consequently in the underlying loading process into extracellular vesicles preferred over the smaller form.

The slower running behaviour in SDS PAGE might be caused by changes in the protein either by posttranslational modifications or by alternative splicing. However, the immunodetection with the HDGF-C antibody did not lead to signals in the respective samples (data not shown).

4.1.5.1.2 Posttranslational modification directs presence in extracellular vesicles

Posttranslational modifications are indeed a tool to denote proteins for exosome mediated transfer. For example some but not all proteins require ubiquitination to be sorted into exosomes [250]. Also other posttranslational modifications seem to play a role for some proteins. There are a few hints that phosphorylation is involved in these sorting processes at least for a few proteins. Mass spectrometry analysis demonstrated that phosphorylated tau proteins were enriched in exosomal fraction compared to the
respectively cell lysates [251]. Furthermore, N-terminal phosphorylation of Annexin A2 is required for sorting into exosomal membranes [252].

The idea of posttranslational modifications as a regulatory element influencing the sorting of HDGF-A into exosomes was supported by another finding. Ectopically expressed HDGF-S133A mutant was repeatedly not present in the exosome fraction indicating that the deleted phosphorylation site at this position might be essential for the sorting process into exosomes (figure 34). Combining exchange of Ser133 to Ala with other Ser to Ala exchanges such as Ser132 to Ala or Ser165 to Ala did not abolish the extracellular vesicle mediated secretion. This indicates that specific patterns of phosphorylation might control the sorting of HDGF-A into extracellular vesicles. However, further experiments are necessary to explore the role of phosphorylation HDGF pattern.

4.1.5.1.3 N-terminus of HDGF-A is critical for localisation in vesicles

The most striking finding was that the novel isoforms HDGF-B and HDGF-C are located at the surface of extracellular vesicles, while the HDGF-A isoform is located within these vesicles (figure 31). Like HDGF-A also endogenous HDGF was found in the lumen of extracellular vesicles, clearly supporting the assumption that the detected endogenous HDGF in the different exosome enriched samples from different celltypes is mainly HDGF-A and not HDGF-B or HDGF-C (figure 33). However, if there is any condition, where one of the HDGF isoforms HDGF-B and -C is expressed that strong, that it can be detected on the surface of extracellular vesicles, remains uncertain.

The presence or absence of the outermost N-terminal amino acid residues seems to control the localisation in or on extracellular vesicles. Not only HDGF-B and HDGF-C but also all N-terminal truncated HDGF-A variants, deleted in 11 or more amino acid residues were found at the surface of extracellular vesicles (figure 35). The missing of the respective peptide seems to be likewise the reason for the surface localisation of HDGF-A S165A, which is reported to be N-terminal truncated [33]. Interestingly, Jeppesen et al. could show that HDGF is associated with the exosomal membrane. However, the applied protocol could not distinguish between intra- and extravesicular localisation [87].

Since extravesicular position was shown for HDGF isoforms -B and -C as well as HDGF-A S165A it seems that the position can be achieved by different modulation ways, alternative splicing on posttranscriptional level or by posttranslational modifications in form of dephosphorylation at position Ser165.

Different proteins have been identified as localised on the exosomal surface such as galectin-5 [253], annexin A2 [252] or Hsp60 [236]. It was proposed that the release of these proteins is mediated by an unconventional pathway including the secretion as free
protein into the extracellular medium, the subsequent binding to lipid rafts present at the plasma membrane followed by internalisation via endocytosis and the final entrance into the MVB. Exosomes, deriving from the respective MVB, carry the protein on their membrane surface [236, 252]. Such a model is also conceivable for the HDGF forms (HDGF-B, HDGF-C and HDGF-A S165A). As indicated in figure 38, the proposed secretion mechanism involves the crossing of the plasma membrane by an unknown alternative secretion mechanism (B1), the binding to lipid rafts present in the plasma membrane (B2), the endocytosis (B3), the maturation to MVBs accompanied by the formation of ILV (B4), and the final release of exosomes carrying the respective lipid rafts decorated with the respective HDGF protein variants (B5).

![Figure 38: Hypothetical model for the secretion mechanisms of HDGF isoforms.](image)

As mentioned earlier the presence of other extracellular vesicles (apoptotic blebs and microvesicles), which derive from the plasma membrane cannot be excluded completely...
and they might be the actual source for the detection of the HDGF variants. Nevertheless, also in this case the corresponding HDGF variants have to cross the plasma membrane in order to end up on the surface of those extracellular vesicles. According to the depletion experiment the mutant HDGF-A S165A as well as HDGF-B and -C are not present as free proteins in the conditioned media. Consequently, on the basis of the proposed secretion model this would require that these proteins bind back with such a high efficiency to the plasma membrane after crossing the membrane so that none or only low levels are released as free protein (B3).

Furthermore, the results of the depletion experiment give rise to the speculation that HDGF-A is released from the cell via an additional alternative pathway, by that HDGF-A ends up as free protein in the extracellular space. Of course it is conceivable that HDGF-A as well as HDGF-B, HDGF-C and the N-terminal truncated HDGF-A use the same pathway in order to cross the membrane, but only the full length HDGF-A does not have the ability to bind back to the cell membrane, probably mediated through inhibition by its specific N-terminus.

Of particular note is that the presence or absence of outermost N-terminal part of HDGF-A is again the critical point as already observed for the interaction behaviour. With respect to the parallels in interaction and secretion behaviour of the different examined HDGF variants, it is reasonably assumed that the altered secretion behaviour is connected to the differential interaction behaviour. The N-terminally altered HDGF variants might be able to bind to a specific protein, which is located at the surface of extracellular vesicles and therefore the actual reason for the differential localisation of those HDGF variants. Conversely, HDGF-A might be sorted into exosomes since by contrast to the other HDGF variants it binds to a specific protein, which is targeted to exosomes. Interestingly, both nucleolin [254] and YB-1 [254] were identified in these vesicles. However, tubulin and actin, binding partners of HDGF-C, are both present in exosomes, too. Therefore, interaction to any protein, which is localised in exosomes, does not directly lead to intraexosomal localisation.

In this context it is interesting that YB-1 is required for the sorting of microRNAs into exosomes [255]. Considering that HDGF-A is interaction partner of YB-1, the interaction depends on the presence of RNA and it is like YB-1 present in exosomes, HDGF-A might adopt a similar function and supports YB-1 in some way.

4.2 N-terminal truncation of HDGF-A

The different experiments regarding interaction and secretion behaviour revealed that the outermost N-terminal amino acid residues are critical for the function of HDGF-A.
Regarding the drastic changes, the N-terminal truncation of HDGF-A appears like an internal switch.

Until now, there has not been any evidence that N-terminal truncation of HDGF-A occurs upon dephosphorylation of Ser165 under physiological conditions, as proposed by Thakar et al. [33], but its discovery would be worthwhile. Intriguingly, N-terminal truncation has been described for HMGB1. Extracellular dipeptidyl peptidase IV cleaves HMGB1 in its N-terminal part and by that abolishes its angiogenic activity. Moreover, DPP-IV inhibition in vivo decreases the levels of truncated HMGB1 [256]. Apart from DPP-IV also thrombin cleaves HMGB1 N-terminally [257].

As suggested by Thakar et al., the N-terminal part of HDGF-A contains a putative MAPK docking site KEYKCGDLVF\textsubscript{17} with 100 % identity to the consensus sequence [33]. Apart from the mentioned changes in secretion and interaction behaviour, truncation would also lead to the loss of this putative site [258]. Moreover, a further really interesting aspect might be that the peptide itself gains a function after cleavage and by that becoming a bioactive peptide.

The Cys12 in HDGF-A appears to influence truncation process in some way because in exosome enriched fractions collected from HDGF C12A expressing cells an additional protein signal with faster electrophoretic running behaviour could be detected, which was sensitive to trypsin digest (figure 36). The enrichment in the exosome containing fraction and moreover the extravesicular localisation corresponded to the results obtained for those HDGF variants, which were either N-terminally truncated in 11 or more amino acid residues (HDGF-A S165A; NΔ11/12 HDGF) or exhibited an alternative N-terminal sequence (HDGF-B and HDGF-C) compared to HDGF-A. This analogy and the lower molecular weight strongly suggested that the detected variant was N-terminally truncated in 11 or more N-terminal amino acid residues.

Thakar et al. demonstrated that an intramolecular disulfide bridge can be formed between Cys12 and Cys108 [33]. On the basis of this observation, deletion of the intramolecular disulfide bridge through the loss of Cys12 might improve the accessibility for the N-terminal truncation mechanism. However, in extracellular vesicles of HDGF C108A overexpressing cells a similar additional truncated fragment was not observed. Since the intramolecular disulfide bridge cannot be formed in this HDGF mutant either, it seems unlikely that the formation or deletion of this disulfide bridge is directly involved in the truncation process.

On the basis of the present data it is difficult to give an explanation for the truncation mechanism. One possibility is that the exchange of Cys12 to an Ala introduces a better motif for the N-terminal truncation mechanism. In addition it is conceivable that with the
exchange of Cys12 to Ala the respective N-terminal peptide adopts a position more accessible for the truncation mechanism. To clarify this further studies are necessary.

4.3 Conclusion and perspectives

One of the major objectives of this thesis was the verification and characterisation of novel HDGF isoforms HDGF-B and HDGF-C. Both were identified to be expressed on mRNA level within this present work and HDGF-C could be also verified as expressed protein by the use of a novel peptide specific antibody. The missing proof of HDGF-B as expressed protein was due to the lack of a fully characterised HDGF-B specific antibody and its verification on protein level remains for future studies. The discovery of the isoforms raises new questions regarding their biological role that can be initially answered with the present study. At the same time the results open various new questions, which need to be responded in future studies.

On the basis of the present results, it becomes obvious that the novel isoforms broaden the functional spectrum of HDGF-A and extend the interactome. Nevertheless, also overlaps regarding function and interaction exist. This includes the common interaction partner Hsc-70 as well as the proliferation activity on cells upon overexpression, which was observed for HDGF-A and HDGF-B but not for HDGF-C.

Exploring the co-precipitates of each isoform revealed quite differential interaction behaviour. The distinct interactions indicate that HDGF-B and -C participate at least partly in different cellular processes than HDGF-A. Only HDGF-B and -C have the ability to co-precipitate the cytoskeleton protein tubulin, whereas HDGF-A does not have this ability. On the one hand it opens new potential functional aspects such as cell migration or cargo transport. In this context further studies need to clarify whether these variants bind directly to tubulin as observed for HRP-3 and which functional consequence can be linked to this interaction. On the other hand this finding is notable because the interaction did not depend on the new alternative N-termini of HDGF-B and -C, but on the lack of the N-terminus of HDGF-A. An opposite result was obtained for the interaction with nucleolins and YB-1. Here the outermost N-terminal amino acid residues of HDGF-A are essential for the interaction with the mentioned proteins, the deletion of them in HDGF-A leads to its loss. In this context, HDGF-B and HDGF-C, missing the respective peptide, are also not able to co-precipitate these proteins. This observation raises the fascinating question in which way the outermost N-terminal peptide can influence the interaction behaviour of HDGF-A, an important question which needs to be answered by further investigations.

It remains unclear whether a direct link can be drawn between differential interactions and the distinct secretion behaviours, but the parallels are obvious. While HDGF-A is located within extracellular vesicles and moreover seems to exit the cell via an additional
pathway, all other HDGF variants (HDGF-B, -C and HDGF-A S165A) with an altered N-terminal part were located at the surface of the extracellular vesicles and could not be found as free protein in the surrounding media. Again N-terminal truncation of HDGF-A is sufficient to induce the alternative secretion behaviour. In theory, this could mean that the cell has two different modes to intervene in secretion and interaction. First, it induces this alternative behaviour in secretion and interaction on posttranscriptional level by the generation of alternative isoforms through alternative splicing. Second, assumed that such a mechanism proposed by Thakar et al. [33] exists and the dephosphorylation at Ser165 in HDGF-A results in N-terminal truncation, the cell can control this on posttranslational level, too.

The subsequent different modes of localisation in the extracellular space might result in different communication pathways. With respect to the generally accepted fact that HDGF is an factor involved in cancer development and progression [40, 45, 50, 57, 62] and the demonstration that HDGF is secreted differentially by extracellular vesicles as an important tool for cancer cells to influence their microenvironment [259], studies in this direction would be a necessary future approach.
5. Literature


82. Bianchi, M. E. (2007) DAMPs, PAMPs and alarmins: all we need to know about danger, J Leukocyte Biol. 81, 1-5.


6. Appendix

6.1 Peptide mass fingerprints of proteins by SDS-PAGE and MALDI-TOF

Table A1: Identification of potential interaction partners by MALDI-TOF mass spectrometry and peptide mass fingerprinting. Spot Number (see figure 20) and the respective identified protein, its Swiss Prot ID as well obtained scoring values are given below.

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<th>Spot No.</th>
<th>Identified in co-precipitate of HDGF</th>
<th>Protein name</th>
<th>Swiss prot ID</th>
<th>Score</th>
<th>Expect</th>
<th>Mass values searched</th>
<th>Mass values matched</th>
<th>Sequence coverage (%)</th>
<th>RMS error ppm</th>
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<td>36</td>
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6.2 SignalP 4.1 prediction results

**HDGF-A**

Figure A1: Prediction result for the presence and location of a putative signal peptide and cleavage site in the HDGF-B amino acid sequence using Signal 4.1 with default settings. The graphical output shows three scores: C (raw cleavage site score), S (signal peptide score) and Y (combined cleavage site score of the C-score and the slope of the S-score) for each position in the sequence. The D-score (discrimination score, combined score of the mean S and the max Y score) is used to discriminate signal peptides from non-signal peptides. The D-cut off was set at 0.45 (default settings).
Figure A2: Prediction result for the presence and location of a putative signal peptide and cleavage site in the HDGF-B amino acid sequence using Signal 4.1 with default settings. The graphical output shows three scores: C (raw cleavage site score), S (signal peptide score) and Y (combined cleavage site score of the C-score and the slope of the S-score) for each position in the sequence. The D-score (discrimination score, combined score of the mean S and the max Y score) is used to discriminate signal peptides from non-signal peptides. The D- cut off was set at 0.45 (default settings).
Figure A3: Prediction result for the presence and location of a putative signal peptide and cleavage site in the HDGF-C amino acid sequence using Signal 4.1 with default settings. The graphical output shows three scores: C (raw cleavage site score), S (signal peptide score) and Y (combined cleavage site score of the C-score and the slope of the S-score) for each position in the sequence. The D-score (discrimination score, combined score of the mean S and the max Y score) is used to discriminate signal peptides from non-signal peptides. The D-cut off was set at 0.45 (default settings).
6.3 Expression plasmids

pcDNA3

Figure A4: Vector Map of pcDNA3 (Invitrogen). The graphical output was obtained from: http://www.snapgene.com/resources/plasmid_files/image_consortium_plasmids.pcDNA3/ (24.04.2016; 18:15).

pEGFP-N3
Figure A5: Vector Map of pEGFP-N3 (Clonetech). The graphical output was obtained from: http://old-site.bioss-freiburg.de/www.bioss.uni-freiburg.de/toolbox/productsf427.html?PL-823&cPath=6_43 (24.04.2016; 18:00)

Figure A6: Vector Map of pEPR-IBA5 (IBA). The graphical output was obtained from: http://search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/IBA_/21905000.20060609.pdf (24.04.2016; 18:30)
7. Erklärung

Versicherung an Eides

Statt Ich, Jessica Nüße,( Lescheder St. 14, 48488 Emsbüren)

versichere an Eides Statt durch meine Unterschrift, dass ich die vorstehende Arbeit selbständig und ohne fremde Hilfe angefertigt und alle Stellen, die ich wörtlich dem Sinne nach aus Veröffentlichungen entnommen habe, als solche kenntlich gemacht habe, mich auch keiner anderen als der angegebenen Literatur oder sonstiger Hilfsmittel bedient habe.

Ich versichere an Eides Statt, dass ich die vorgenannten Angaben nach bestem Wissen und Gewissen gemacht habe und dass die Angaben der Wahrheit entsprechen und ich nichts verschwiegen habe.

Die Strafbarkeit einer falschen eidesstattlichen Versicherung ist mir bekannt, namentlich die Strafandrohung gemäß § 156 StGB bis zu drei Jahren Freiheitsstrafe oder Geldstrafe bei vorsätzlicher Begehung der Tat bzw. gemäß § 161 Abs. 1 StGB bis zu einem Jahr Freiheitsstrafe oder Geldstrafe bei fahrlässiger Begehung.

__________________________________________  __________________________
Ort, Datum                                   Jessica Nüße
8. Danksagung

Es war eine aufregende, anstrengende aber auch schöne Zeit. Und ich schulde vielen Menschen großen Dank, die mich während dieser Zeit in ganz unterschiedlicher Weise unterstützt, kritisiert, gelobt, auch mal abgelenkt haben und mich mit ganz viel Kaffee versorgt haben.

Als erstes muss ich mich bei Dr. Frank Dietz bedanken, der mich in dieser ganzen Zeit betreut, mich in jeglicher Hinsicht unterstützt hat und nie den Glauben daran verloren hat, dass ich das schaffe (und das obwohl ich das nie so wirklich geglaubt habe). Frank, großen Dank für die viele Zeit, die du für mich hattest, für das Vertrauen, was du in mich gesetzt hast und die Unterstützung in allen Situationen. Ohne deine große Zuversicht wäre es wesentlich schwieriger gewesen, die Arbeit bis zum Ende durchzuziehen. Auch bedanke ich mich für die Korrektur meiner Arbeit und für die vielen wertvollen Tipps. Es war toll, mit dir zusammen zu arbeiten und über HDGF und seinen möglichen (und unmöglichen) Funktionen zu fachsimpeln.

Als zweites möchte ich mich bei Herrn Professor Dr. Sörge Kelm bedanken, der mir überhaupt erst die Möglichkeit für diese Arbeit gegeben hat, indem er mich in seine Arbeitsgruppe aufgenommen hat und mir stets mit Rat und Tat zur Seite stand. Auch Dr. Mirastshijski gebührt an dieser Stelle großen Dank, die mich während der zweiten Hälfte dieser Arbeit als wissenschaftliche Mitarbeiterin beschäftigt hat.

Ein weiteren großen Dank gebührt Herrn Prof Dr. Stick und Herrn Dr. Franken, die sich dazu bereit erklärt haben, die Beurteilung dieser Arbeit zu übernehmen. Vielen Dank! Prof. Dr. Dotzauer, Dr. Franken, Dr. Peters, Prof. Dr. Kelm, Judith und Johannes danke ich dafür, dass sie sich die Zeit nehmen und das Kolloquium stellen.

Bei der gesamten Arbeitsgruppe Nazila, Petra B., Petra S., Sabine, Judith, Mario, Thaddeus, Tanja, Veronika, Claudine, Frank, Janina und vielen anderen bedanke ich mich für das wirklich tolle Arbeitsklima und für die zahlreichen Tipps und Hilfestellungen. Ich bin immer gern zur Arbeit gefahren und vermisse die Arbeit mit euch!

Dr. Janina Oetjen danke ich für die Möglichkeit und der Hilfe mit der MALDI-TOF-MS Analyse. Dr. Federico Paroni danke ich ganz herzlich für die Einweisung und Unterstützung bei der Real-time PCR. Du hast dir für all meine Fragen stets Zeit genommen, was nicht selbstverständlich ist. Der AG Friedrich und insbesondere Arjinka, vielen Dank für die Hilfe mit der Ultrazentrifuge und dem Apotome.

Ich möchte mich weiterhin bei allen bedanken, die mir diese Arbeit überhaupt ermöglicht haben: Ein ganz besonderer Dank geht an meine Eltern, die mir das Studium ermöglichten und mir auch während der Anfertigung der Doktorarbeit immerzu
Meiner lieben Schwester Henni und meinen tollen Freunden danke ich für die Zeit, Zuspruch und Geduld, womit sie mir stets zur Seite standen und mich immer wieder aufgemuntert haben und für Ablenkung gesorgt haben.
Meinem Freund Christian danke ich aus ganzem Herzen für alles.