Functional characterization of the placenta specific protein PLAC1 and its use for cancer immunotherapy

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Diana Barea Roldán
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Nunca desistas de un sueño.
Sólo trata de ver las señales que te lleven a él.

Never desist from a dream.
Just try to see the signs that lead you to it.

Paulo Coelho

Summary

Antibody-based cancer therapies have been successfully introduced into the clinic and have emerged as the most promising therapeutics in oncology. The limiting factor regarding the development of therapeutical antibody vaccines is the identification of tumor-associated antigens. PLAC1, the placenta-specific protein 1, was categorized for the first time by the group of Prof. Sahin as such a tumor-specific antigen.

Within this work PLAC1 was characterized using a variety of biochemical methods. The protein expression profile, the cellular localization, the conformational state and especially the interacting partners of PLAC1 and its functionality in cancer were analyzed.

Analysis of the protein expression profile of PLAC1 in normal human tissue confirms the published RT-PCR data. Except for placenta no PLAC1 expression was detectable in any other normal human tissue. Beyond, an increased PLAC1 expression was detected in several cancer cell lines derived of trophoblastic, breast and pancreatic lineage emphasizing its properties as tumor-specific antigen.

The cellular localization of PLAC1 revealed that PLAC1 contains a functional signal peptide which conducts the propeptide to the endoplasmic reticulum (ER) and results in the secretion of PLAC1 by the secretory pathway. Although PLAC1 did not exhibit a distinct transmembrane domain, no unbound protein was detectable in the cell culture supernatant of overexpressing cells. But by selective isolation of different cellular compartments PLAC1 was clearly enriched within the membrane fraction. Using size exclusion chromatography PLAC1 was characterized as a highly aggregating protein that forms a network of high molecular multimers, consisting of a mixture of non-covalent as well as covalent interactions. Those interactions were formed by PLAC1 with itself and probably other cellular components and proteins. Consequently, PLAC1 localize outside the cell, where it is associated to the membrane forming a stable extracellular coat-like structure.

The first mechanistic hint how PLAC1 promote cancer cell proliferation was achieved identifying the fibroblast growth factor FGF7 as a specific interacting partner of PLAC1. Moreover, it was clearly shown that PLAC1 as well as FGF7 bind to heparin, a glycosaminoglycan of the ECM that is also involved in FGF-signaling. The participation of PLAC1 within this pathway was approved after co-localizing PLAC1, FGF7 and the FGF7 specific receptor (FGFR2IIIb) and identifying the formation of a trimeric complex (PLAC1, FGF7 and the specific receptor FGFR2IIIb). Especially this trimeric complex revealed the role of PLAC1. Binding of PLAC1 together with FGF7 leads to the activation of the intracellular tyrosine kinase of the FGFR2IIIb-receptor and mediate the direct

phosphorylation of the AKT-kinase. In the absence of PLAC1, no FGF7 mediated phosphorylation of AKT was observed. Consequently the function of PLAC1 was clarified: PLAC1 acts as a co-factor by stimulating proliferation by of the FGF7-FGFR2 signaling pathway.

All together, these novel biochemical findings underline that the placenta specific protein PLAC1 could be a new target for cancer immunotherapy, especially considering its potential applicability for antibody therapy in tumor patients.

Zusammenfassung

Antikörper-basierte Krebstherapien gehören zu den vielversprechendsten Therapieformen in der Onkologie. Der limitierende Faktor für die Entwicklung therapeutischer Antikörper-Impfstoffe ist die Identifizierung Tumor-assoziierter Antigene. PLAC1, Plazenta-spezifisches Protein 1, wurde erstmals in der Gruppe von Prof. Sahin als solch ein tumorspezifisches Antigen kategorisiert.

Im Rahmen dieser Arbeit wurde PLAC1 anhand einer Vielzahl von biochemischen Methoden charakterisiert. Das Protein- Expressionsprofil, die zelluläre Lokalisation, die strukturelle Konformation des Proteins in der Zelle und vor allem das Interaktionsnetzwerk von PLAC1 sowie dessen Funktion wurden analysiert.

Die Analyse des Protein-Expressionsprofil von PLAC1 auf menschlichen Gewebeproben und menschlichen Krebszelllinien zeigt eindeutig, dass die PLAC1 Expression ausschließlich auf Plazenta beschränkt ist. Darüber hinaus wurde eine erhöhte PLAC1 Expression in Trophoblasten-, Brust- und Bauchspeicheldrüsenkrebs abstammen Krebszelllinien detektiert, was wiederum die Kategorisierung als tumorspezifisches Antigen bestätigt.

Analysen bestätigten, dass PLAC1 ein funktionelle Signalpeptid (SP) enthält. Dieses Signalpeptid ist für die Sezernierung von PLAC1 via ER (endoplasmatisches Retikulum) und den sekretorischen Weg verantwortlich. Obgleich PLAC1 keine Transmembrandomäne aufweist, konnte kein ungebundenes Protein im Zellkulturüberstand überexprimierender Zellen nachgewiesen werden. Jedoch konnte PLAC1 nach Isolierung verschiedenster zellulärer Kompartimente eindeutig in der Membranfraktion angereichert werden. Mittels Größenausschlusschromatographie (Gelfiltration) konnte PLAC1 als aggregierendes Protein charakterisiert werden, welches ein Netzwerk hochmolekularer Multimere bildet. Diese PLAC1-Multimere bestehen aus einer Mischung nicht-kovalenter als auch kovalenter Wechselwirkungen von PLAC1 Monomeren und vermutlich auch anderer zellulärer Komponenten und Proteinen. Folglich lokalisieren PLAC1 außerhalb der Zelle, wo es an die Membran unter Bildung eines stabilen extrazellulären Struktur zugeordnet ist.

Wie PLAC1 die Proliferation von Krebszellen zu fördern scheint, wurde durch die Identifizierung des Fibroblasten-Wachstumsfaktor FGF7 als spezifischen Interaktionspartner von PLAC1 aufgeklärt. Es konnte nachgewiesen werden, dass PLAC1 ebenso wie FGF7 an Heparin bindet, welches ebenso wie FGF7 auch an der FGF-Signalkaskade beteiligt ist. Die Beteiligung von PLAC1 an dieser Signalkaskade wurde letztlich durch die Co-Lokalisation von PLAC1, FGF7 und die FGF7 spezifischen Rezeptor (FGFR2IIIb) sowie die Bildung eines trimeren Komplex (PLAC1, FGF7 und der spezifische Rezeptor FGFR2IIIb) bestätigt.

Speziell anhand des trimeren Komplexes konnte die Rolle von PLAC1 zeigte werden. Die Bindung von PLAC1 zusammen mit FGF7 an den FGF-Rezeptor führt zu dessen Aktivierung und vermittelt die direkte Phosphorylierung der AKT-Kinase. In Abwesenheit von PLAC1 wurde keine FGF7 vermittelte Phosphorylierung von AKT beobachtet. Demnach konnte die molekulare Rolle von PLAC1 bezüglich der zellulären Prozesse geklärt werden: PLAC1 fungiert als Co-Faktor des FGF7-FGFR2 induzierten Signalwegs, welcher unter anderem für die Regulation des zellulären Wachstums verantwortlich ist.

Insgesamt unterstreichen diese neuartigen biochemischen Erkenntnisse, dass das Plazenta-spezifische Protein PLAC1 ein fantastisches neues Zielantigen für die Krebs-Therapie ist, insbesondere angesichts dessen potentiellen Anwendbarkeit für die Antikörper-Therapie bei Tumorpatienten.

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ABBREVIATIONS

^{35}S radioactive sulfur isotope of mass number 35

°C degree centigrade

µ micro

Ig immunoglobulin

A ampere

APS ammonium persulfate

aa amino acid

BrdU 5-Brom-2'-desoxyuridine

BSA bovine serum albumin (Fraction V)

bp base pair

Ci curie

C-Terminus carboxy-terminus

CIAP calf intestine alkaline phosphatase

DNA deoxyribonucleic acid

ds double-stranded

Da Dalton

ddH_2O dist water

DMEM Dulbecco's Modified Eagles medium

DMSO dimethyl sulfoxide

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

eGFP enhanced green fluorescence protein

ER endoplasmic reticulum

ECL enhanced chemoluminescence

E.coli Escherichia coli

F(ab') antigen binding antibody fragment

FPLC fast protein liquid chromatography

FCS fetal calf serum

Fc constant domain antibody fragment

FACS florescent activated cell sorting

Fig. figure

g gram

HA hemagglutinin

HPLC high pressure liquid chromatography

h hour(s)

IP immunoprecipitation

IPTG isopropyl-β-thiogalactopyranoside

l liter

LB Luria-Bertani-medium

m mili

M molar (mol/l)

mAb monoclonal antibody

MAGE melanoma antigen

min minute(s)

mRNA messenger RNA

N-Terminus amino-terminus

n nano

NEAA non-essential aminoacids

NTP nucleotide triphosphate

OD optical density

PBS phosphate buffered saline

ORF open reading frame

pH negative decadic logarithm of proton concentration

pDNA plasmid DNA

PAGE polyacrylamid gel electrophorese

PCR polymerase chain reaction

RIPA radio immunoprecipitation assay

rpm revolutions per minute

RT room temperature

SDS sodium dodecyl sulfate

SDS-PAGE discontinuous SDS-polyacrylamide gel electrophoresis

s second(s)

TEMED N,N,N',N'-tetramethylethylenediamine

Tris tris-(hydroxymethyl)-aminomethane

Triton X-100 p-isooctylphenylpolyoxyethylen

Tween-20 polyoxyethylene (20) sorbitan monolaurate

V volt

v/v volume per volume

w/v weight per volume

LETTER CODE FOR AMINO ACIDS

A Alanine

C Cysteine

D Aspartic acid

E Glutamic acid

F Phenylalanine

G Glycine

H Histidine

I Isoleucine

K Lysine

L Leucine

M Methionine

N Asparagine

P Proline

Q Glutamine

R Arginine

S Serine

T Threonine

V Valine

W Tryptophan

Y Tyrosine

I INTRODUCTION

1.1 Characteristics of human cancer

Cancer is the major health problem worldwide and one of the most important causes of morbidity and mortality the industrialized countries (1). Cancer is characterized by abnormal growth of cells which tend to proliferate in an uncontrolled way, invading and thereby destroying adjacent tissues. In some cases these abnormal cells can invade adjoining parts of the body and spread to other organs via lymph or blood (metastatic spread) (2). This process is referred to as metastasis. Metastases are the major cause of death from cancer. Cancer is not one disease. It is a group of more than 200 different and distinctive diseases, since cancer can involve any tissue of the body and have many different forms in each body area. The cancer-forming process, called oncogenesis or tumorgenesis, is an interplay between genetics and the environment (3). The mechanisms of carcinogenesis in humans are often multifactorial and complex. Different factors may act by different mechanisms and at different stages of tumor development (4). Cancer is one of the most frequent causes of death in adults, followed by infectious diseases and cardiovascular diseases. According to a study performed by the world health organization (WHO) cancer was a leading cause of death worldwide in the past years. 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide (Figure 1), with 56% of the cases and 64% of the deaths in the economically developing world (5, 6).

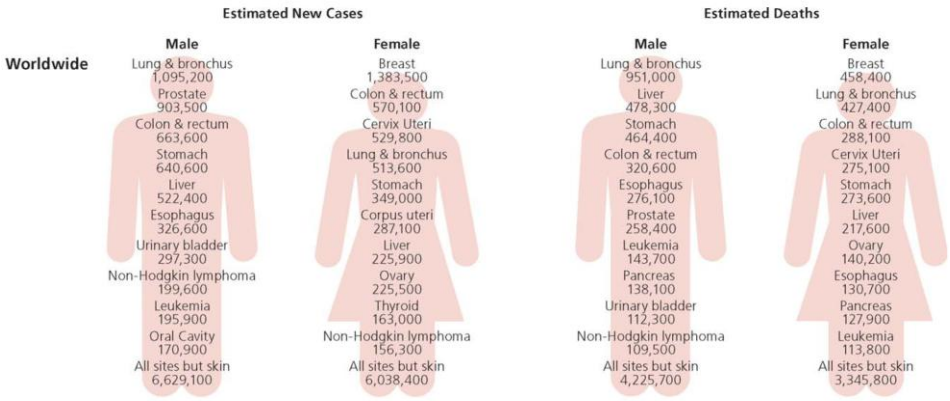

Figure 1. Estimated new cancer cases and deaths worldwide for leading cancer sites.
About 13% of all cancer cases and 8% cancer deaths were estimated to have occurred in 2008 worldwide, with 56% of the cases and 64% of the deaths in the economically developing world. The most frequently diagnosed cancers and the leading cause of cancer death are breast cancer in females and lung cancer in males (5).

The main types of cancer are: lung (1.4 million deaths), stomach (740 000 deaths), liver (700 000 deaths), colorectal (610 000 deaths) and breast (460 000 deaths). Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030 (7). The frequency of a particular cancer may differ between men and women. While skin cancer is the most common type of cancer for both men and women, the second most common type in men is prostate cancer and in women breast cancer. Today most cancers can be treated by conventional tumor therapies like surgery, radiotherapy and chemotherapy. However, as an alternative modality of treatment novel therapies like gene therapy and immunotherapy as well as the implementation of individualized therapy were significantly developed during the last decades (8). Especially regarding the cancer immunotherapy successful results were achieved and have thereby generated a lot of interest and research activity in immunology (9).

1.2 Antibody based cancer immunotherapy

Immunotherapy is one of the most elegant concepts in cancer therapy. The central idea is based on recruiting and restoring reactivity of the host´s immune system to combat cancer (9). Immunotherapy of cancer began about more than one hundred years ago, when Dr. William Coley showed that treatment of cancer patients by unspecific stimulating the immune system with a mixed vaccine of bacteria lead to recovery (10). Many years of research have finally produced successful examples of immunotherapy for cancer. Especially antibody therapeutics lead to high success rates, so that antibodies are emerging as one of the major class of therapeutic agents in the treatment of many human diseases, in particular in cancer (5, 11). Antibodies are a crucial component of the immune system. Their specific binding to an antigen expressed on the surface of a cell lead to the activation of the immune systems so that the marked cell gets subsequently destroyed. In the context of cancer immunotherapy, monoclonal antibodies can react against antigens selectively expressed on cancer cells, leading to the destruction of those cancer cells (12). To date, 31 mAb have been approved by the United States Food and Drug Administration (FDA) for clinical applications. In addition, several hundreds of mAb are being developed clinically by many biotech and pharmaceutical companies for various disease indications (11, 13-15). Some of them are listed in Table 1.

Table 1. Monoclonal antibodies approved for therapeutic use

Generic name	Trade name	Antigen	Approved indication	FDA	Sponsor
Muromomab	Orthoclone	CD3	Allograft rejection in allogeneic renal transplantation	6/19/86	Ortho Biotech Inc
Abciximab	Reopro	GPIIb/IIIa receptor	Prevention of cardiac ischemic complications	12/16/93	Centocor
Rituximab	Rituxan	CD20	Non-Hodgkin's Lymphoma, chronic lymphocytic leukemia and rheumatoid arthritis	11/26/97	Genentech and Biogen Idec
Daclizumab	Zenapax	IL-2Ra	Prophylaxis of acute organ rejection in renal transplants	12/10/97	Hoffman-La Roche
Basiliximab	Simulect	IL-2Ra	Prophylaxis of acute organ rejection in renal transplantation	5/12/98	Novartis
Palivizumab	Synagis	RSV F protein	Respiratory syncytial virus infection	6/19/98	Medimmune
Infliximab	Remicade	TNFa	Crohn's disease and rheumatoid arthritis etc	8/24/98	Centocor
Trastuzumab	Herceptin	Her2	Breast cancer	9/25/98	Genentech
Gemtuzumab	Mylotarg	CD33	Acute myeloid leukemia	5/17/00	Wyeth/Pfizer
Alemtuzumab	Campath	CD52	B-cell chronic lymphocytic leukemia	5/7/01	Ilex/Genzyme
Ibritumomab	Zevalin	CD20	B-cell non-Hodgkin's lymphoma	2/19/02	Biogen
Adalimumab	Humira	TNFa	Rheumatoid arthritis and Crohn's disease etc	12/31/02	Abbott
Omalizumab	Xolair	IgE	Moderate to severe persistent asthma	6/20/03	Genentech
Tositumomab	Bexxar	CD20	Non-Hodgkin's lymphoma	6/27/03	Corixa/GSK
Efalizumab	Raptiva2	CD11a	Moderate to severe plaque psoriasis	10/27/03	Genentech
Cetuximab	Erbitux	EGFR	Head and Neck cancer, colorectal cancer	2/12/04	ImClone/BMS/ Merck kGa
Bevacizumab	Avastin	VEGF-A	Various solid tumors	2/26/04	Genentech
Natalizumab	Tysabri	a4-integrin	Multiple sclerosis and Crohn's disease	11/23/04	Biogen Idec/Elan
Ranibizumab	Lucentis	VEGF-A	Age-related macular degeneration	6/30/06	Genentech
Panitumumab	Vectibix	EGFR	Metastatic colorectal carcinoma	9/27/06	Amgen
Eculizumab	Soliris	C5	Paroxysmal nocturnal hemoglobinuria	3/16/07	Alexion
Certolizumab	Cimzia	TNFa	Crohn's disease and rheumatoid arthritis	4/22/08	UCB, Inc
Golimumab	Simponi	TNFa	Rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis	4/24/09	Centocor Ortho Biotech
Canakinumab	Ilaris	IL-1b	Cryopyrin-associated periodic syndromes	6/17/09	Novartis
Ustekinumab	Stelara	IL-12/ IL-23	Plaque psoriasis	9/25/09	Centocor Ortho Biotech
Ofatumumab	Arzerra	CD20	Chronic lymphocytic leukemia	10/26/09	Glaxo Grp Ltd
Tocilizumab	Actemra	IL-6R	Rheumatoid arthritis	1/8/10	Roche/Chugai
Denosumab	Prolia	RANK ligand	Postmenopausal women with risk of osteoporosis	06/02/10	Amgen
Catumaxomab	Removab	EpCAM and CD3	Intraperitoneal treatment of malignant ascites in patients with EpCAM-positive carcinomas		TRION Pharma
Edrecolomab	Panorex	EpCAM	Colon cancer		Wellcome/Centocor
I131-TNT	Cotara	DNA	Lung cancer		MediPharm Biotech
Nimotuzumab	Theracim5	EGFR	Nasopharyngeal carcinomas and head and neck tumors		CIM/CIMAB/ YM Bioscience

Approved therapeutics are listed with respective trade names, antibody names, antigen, proposed mechanisms of action and approved indications. Products are listed in order of first regulatory approval, from Orthoclone OKT3 (1986) to Tysabri (2004). ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; EGFR, epidermal-growth-factor receptor; gp, glycoprotein; K_d, antigen-binding affinity; RSV, respiratory syncytial virus; TNF, tumor-necrosis factor; VEGF, vascular endothelial growth factor. (14)

Monoclonal antibodies (mAb) achieve their therapeutic effect through various mechanisms (16). They can have (i) direct effects in producing apoptosis or programmed cell death. They can (ii) act as antagonist or agonists of the target molecule by blocking growth factor receptors, inhibiting proliferation of tumor cells. And they can (iii) have indirect effects recruiting cells that have cytotoxicity, such as monocytes and macrophages. This type of antibody-mediated cell killing is called antibody-dependent cell mediated cytotoxicity (ADCC). Moreover monoclonal antibodies also bind complement, leading to direct cell toxicity, known as complement dependent cytotoxicity (CDC) (9, 16). Moreover, antibodies are also used as vehicles to specifically deliver a covalently linked cytotoxic agent to the tumor (17-19).

1.3 Identification of target structures for antibody therapy

The success of any cancer vaccine relies on the induction of an effective tumor-specific immune response. In the context of antibody based cancer immunotherapy, monoclonal antibodies react against antigens selectively expressed on cancer cells, so called tumor-selective cell surface proteins or tumor antigens. Mostly those antigens are critical for the malignant phenotype of a cancer cell. The identification of tumor-associated antigens represents the major challenge of tumor immunologists especially regarding the development of therapeutic antibody vaccines. The requirements for a target structure for antibodies are (11, 12):

- overexpressed by tumor cells
- no or restricted expression in normal tissue
- stable expression in the primary tumor and/or in metastasis
- should be accessible to antibodies
- should have a define cellular function, that might be involved in tumorgenesis

The molecular identification of cancer antigens has opened new possibilities for the development of effective immunotherapy for patients with cancer. Today, most of the known tumor antigens were placenta- or germline- derived gens.

1.3.1 Cancer-germline antigens as targets against cancer

An ideal cancer antigen for immunotherapy would be selectively and stably expressed by the tumor, absent from normal tissues, and crucial for the survival of the cancer cell. Most of these antigens are cancer-germline genes (CG; also known as cancer-testis) (20-23). Based on their immunogenicity in cancer patients and restricted expression, cancer-germline antigens are the ideal targets for therapeutic cancer vaccines (24-28). They clearly represent the most specific tumor antigens discovered to date. More than 40 CG-antigen families have been identified so far. These antigens are encoded by genes that are normally expressed only by gametes (oocytes and spermatocytes) and trophoblastic cells (cells that contribute to the formation of the chorion and the placenta) (29). Several characteristics of CG-antigens known to date are summarized in Table 2.

Table 2. Characteristics of cancer-germline genes

Expression restricted to gametogenic tissues (ovary and testis) and cancer
Coding genes frequently map to chromosome X
Often exist as multigene families
Heterogeneous expression in cancer: genes are silent in normal tissues but activated in cancer
Immunogenic in cancer patients: induce spontaneous T cell or antibody responses
Gene transcription is associated with promoter demethylation: activation by hypomethylation and/or histone deacetylase inhibitors *in vitro*
Expression may be associated with tumor progression and with tumors of high metastatic potential

Induction of CG-gene expression in cancer is related to demethylation of specific promoter region (CpG islands). In normal somatic cells CG-genes were not expressed since CpG islands at promoters were methylated (30-32). During tumorgenesis DNA hypomethylation is frequently observed: CG-gene promoters become demethylated in cancer cells (33). Demethylation leads to the expression of CG-genes. Nevertheless, cancer-associated demethylation alone is not sufficient for the induction of CG-gene expression (34, 34-36). It has been postulated that the expression of CG-antigens in cancer may be the consequence of induction or activation of a gametogenic program in cancer (24). There are many similarities between cancer cells and gametes or trophoblasts. They share properties like immortalization, invasion, lack of adhesion, angiogenesis and demethylation. According to this hypothesis, the different CG expression profiles seen in cancer may correspond to the profiles of CG-antigens

normally expressed during different stages of gametogenesis or trophoblast development (26, 29). However, CG-genes are expressed in tumors with a low frequency (about 40%) (37). To address the largest possible number of patients, it is important to identify new antigens. At this time, several CG-antigens, e.g. MAGE3 and NY-ESO-1, are evaluated in clinical trials as candidates for immunotherapy (38-40).

1.3.2 Placenta and cancer: placenta- specific antigens

The similar biological features of trophoblast and cancer cells were already observed by the Scottish embryologist John Beard in the last century. In 1902 he proposed "The trophoblastic theory of cancer" (*John Beard*, 1857-1924) (41, 42). In his publication he drew attention to the fact that when the placenta implants into the uterus, the way it invades the mother´s tissue is analog to cancer (41, 42). Today, many common features between placental and cancers have been observed (43-45):

- like tumor cells, trophoblast cells have a very high proliferative capacity and exhibit molecular characteristics found in rapidly dividing cancer cells (46-48);
- cancer cells spread throughout the host, similar to trophoblast cells that migrate and invade the uterus (49-52);
- molecular circuits involved in neoangiogenesis are also likely shared between trophoblast and cancer cells (53-56);
- cancer and placenta have the ability to modulate the host immune response (57, 58);
- regulation of telomerase activity is also a common feature (45, 59)
- chromosomal aneuploidy has been observed in trophoblast- and tumor- cells (60, 61)
- and epigenetic alterations seems to be implicated in the placenta formation and cancer development (62, 63).

However, the selective advantage for tumors to acquire these placenta specific characteristics might be due to the expression of placenta-restricted genes (e.g. CG-antigen) (23, 64, 65). The idea that trophoblastic traits expressed by cancer cells mediate their malignant phenotype, emphasize the necessity to identify and characterize placenta-tumor specific antigens (66, 67).

1.4 The placenta specific protein PLAC1 is a new member of the CT-antigens

High throughput gene expression profiling as well as *in silico* identification techniques were used to identify genes highly expressed in placenta and different cancer types, but with a relatively restricted expression in normal tissues. Using these methods the placenta specific protein PLAC1 was identified (68).

1.4.1 Genomic identification of the placenta specific protein PLAC1

The placenta specific protein PLAC1 was described by Cocchia et al. as an X-linked gene with restricted expression to cells from trophoblast lineage (69). The PLAC1 gene localizes to a region of the X chromosome near the HPRT (Hypoxanthin-Phosphoribosyl-Transferase 1) locus (Xq26). This region is supposed to be important during placental and fetal development, as large chromosomal deletions up to approximately 10cM in length around the HPRT locus result in fetal growth retardation and neonatal death (70). The PLAC1 gene encodes six exons: The first five exons are relatively small 89bp, 86bp, 152bp, 156bp and 72bp and the last exon, containing the entire open reading frame (ORF) is the largest one, encompassing 889 bp (71).

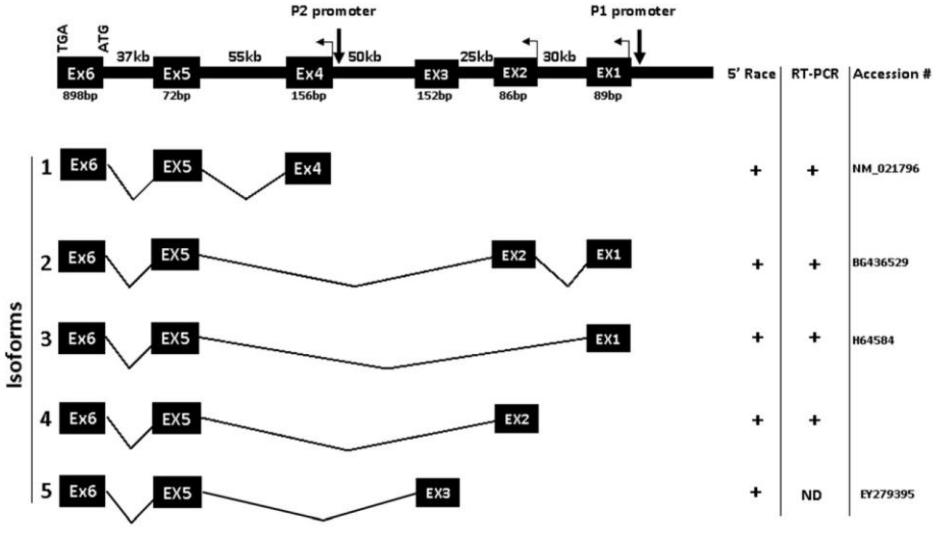

Figure 2. Schematic representation of human PLAC1 gene structures with dual promoters.
The top line illustrates the positions of promoters P1 and P2 in relation to six exons, introns and their sizes. Five human splice transcript isoforms detected by RACE and RT-PCR are diagrammed below. For human only transcripts starting at P2 promoter include Exons 4-6; transcripts originating at P1 promoter include various other exons but always skip exon 4. The columns at right indicate isoforms detected from a human placental RNA library or in BeWo cells by 5´-RACE experiments or RT-PCR. Available Genbank database accession numbers supporting isoforms are also indicated (71).

Moreover, the PLAC1 gene contains two different transcription start sides: promoter P1 and P2. The resulting intron-exon structures give rise to five alternative isoforms, which are diagrammed in Figure 2. Transcripts starting at P1 may include exons 2 or 3 but always skip exon 4, whereas transcripts originating at P2 always include exons 4, 5 and 6; all transcripts include the sole coding sequence of exon 6 (71).

1.4.2 The expression of PLAC1 is restricted to the placenta

Northern analysis and *in situ* hybridization revealed that PLAC1 mRNA expression is restricted to placenta. No detectable expression of PLAC1 mRNA was observed in other normal adult or fetal tissues (69, 72). Furthermore, PLAC1 mRNA expression was restricted to the cells of trophoblastic origin, specially limited to trophoblasts of the chorionic villi. No expression was detected within the decidua or extracellular trophoblast (72). These observations were confirmed by quantitative reverse transcriptase polymerase chain reactions (RT-PCR) but also demonstrated expression at much lower levels in testis (68, 73).

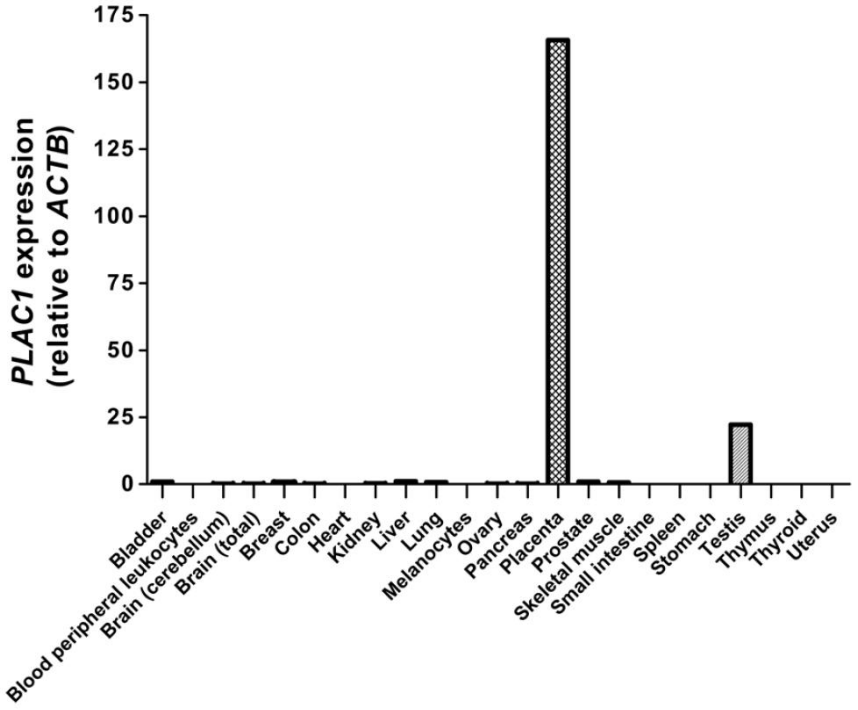

Figure 3. PLAC1 expression in normal tissues.
Quantitative real-time RT-PCR was performed with cDNA samples prepared from RNAs from several normal tissues. ACTB was used as an endogenous control. The cDNA templates used were normalized on the basis of ACTB amplification. Normal bladder was used as reference polymerase chain reactions (RT-PCR) but also demonstrated expression at much lower levels in testis (68, 73).

Further, immunohistochemical as well as biochemical approaches were used to precise the subcellular localization of PLAC1 within the placenta (74). Fant and colleges showed that PLAC1 localized at the apical region of the syncytiotrophoblast in proximity to the microvillus membrane surface (Figure 4). Moreover, PLAC1 was only observed to localize at the maternal-facing microvillus membrane surface of the syncytiotrophoblast (MVM) but not to the fetal-facing basal surface. These observations lead to the assumption that PLAC1 is a membrane-associated protein (74).

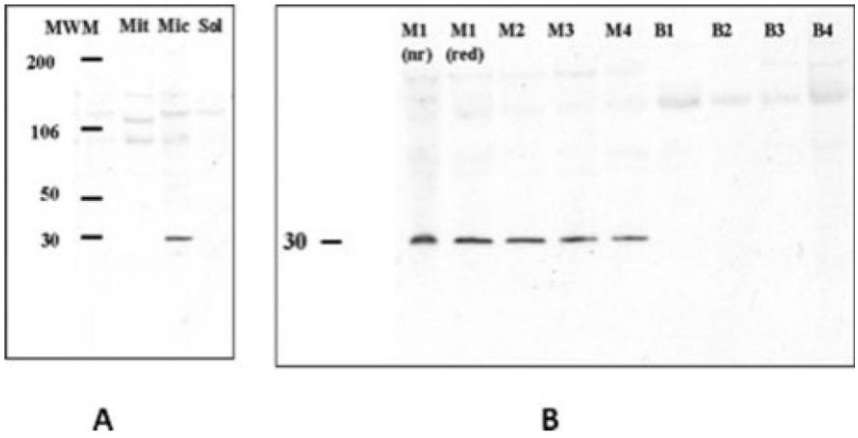

Figure 4. Subcellular distribution of PLAC1 immunoreactivity.
Subcellular fractionation followed by differential centrifugation was utilized to assess PLAC1 distribution among the major intracellular compartments. In addition, plasma membranes enriched specifically from the maternal-facing microvillus membrane surface of the syncytiotrophoblast (MVM) and the fetal-facing basal surface of the syncytiotrophoblast membrane (BM) were also examined. Subcellular and plasma membrane fractions derived from placental tissue were subjected to immunoblotting analysis. Panel A: Subcellular fractions obtained from whole placenta. Mic= microsomal fraction; Mit= mitochondrial fraction; Sol= soluble fraction. Panel B: Membrane fractions enriched for distinct regions of the differentiated syncytiotrophoblast; M= apical, microvillus membrane surface; B=basal membrane surface. (74)

1.4.3 Characteristic features of the placenta specific protein PLAC1

The human ORF encodes a putative protein of 212 amino acids (aa). This putative protein encodes a cleavable signal peptide of 23 amino acids (SignalP) and is predicted to localize extracellular with a probability of 56% (PSORT). Further, bioinformatical characterization predict a transmembrane (TM) domain at the N-terminus immediately downstream of the signal peptide (aa 23-40), four possible glycosylation sites as well as thirteen phosphorylation sites (75). Interestingly, the placenta specific protein PLAC1 is highly conserved over different species as shown in Figure 5.

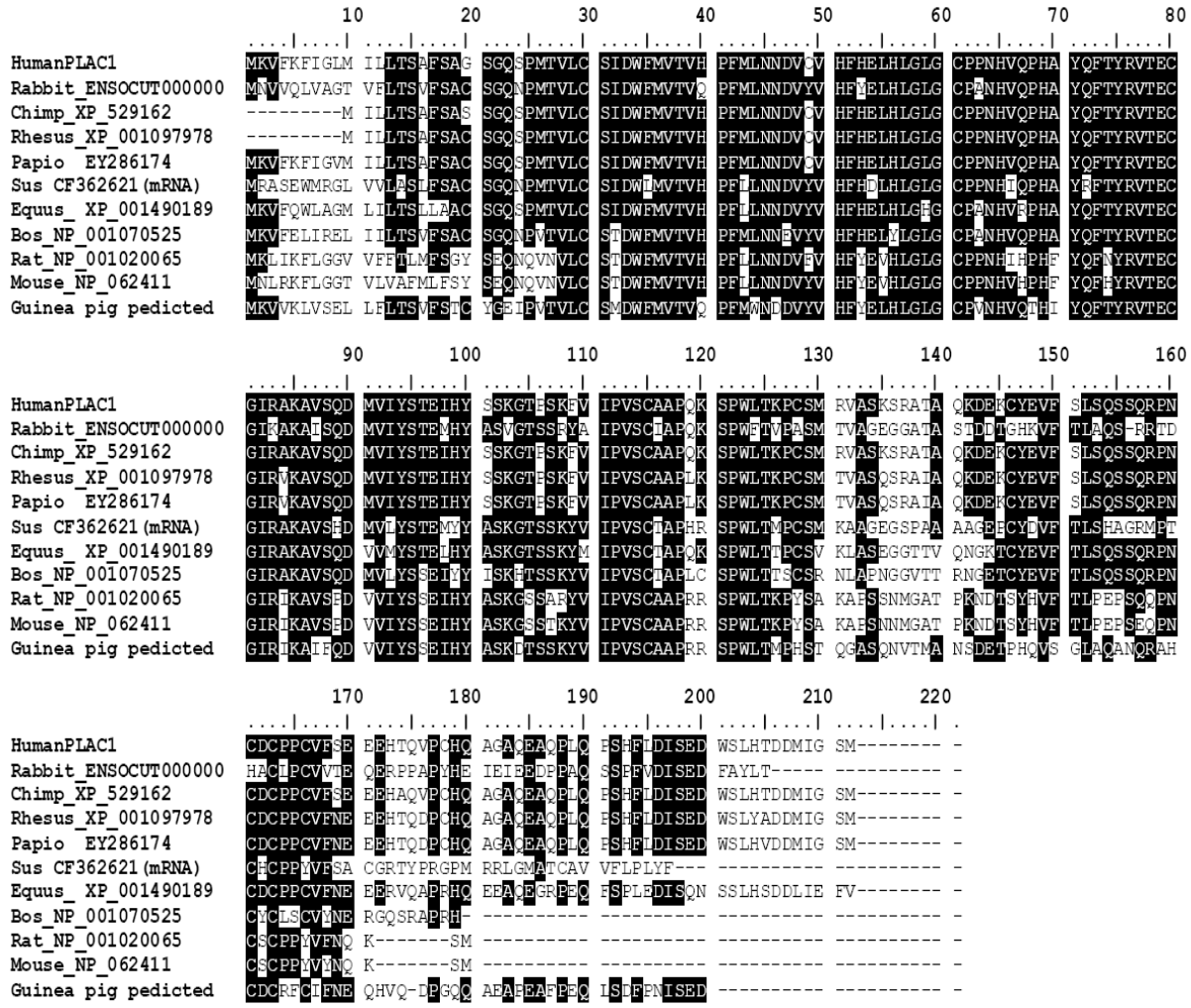

Figure 5. The PLAC1 sequence is highly conserved within different species.
Multiple sequence alignment using PLAC1 amino acid sequences descending from different species were aligned with the public available web server http://www.ebi.ac.uk/Tools/msa/clustalw2/.

In addition, the number of cysteine residues is considerable: human PLAC1 encodes eleven cysteine, in contrast to mouse PLAC1 which encodes six cysteine. However, the conserved cysteine residues are suggested to be involved in forming disulfide bridges, similar to *zona pellucida*-N (ZP-N) subdomains (76). Approximately 30% homology was detected for human PLAC1 with the ZP3 (*zona pellucida* 3) protein. ZP3 proteins are specific sperm-binding glycoproteins also referred to as sperm receptors in the *zona pellucida*. The *zona pellucida* (ZP) is a specialized extracellular matrix that surrounds the oocyte and early embryo. It is composed of four glycoproteins (ZP1-4) with various functions during oogenesis, fertilization and preimplantation development (77, 78). Four of six cysteine residues within the ZP-N domain of human and murine PLAC1 are conserved in different species and correspond to

cysteine residues 1–4 of the ZP domain (79). The presence of the ZP3 motif has also been recognized in various extracellular proteins, i.e. transforming growth factor (TGF-beta) receptor (80). ZP proteins consist of two subdomains: the N-terminal (ZP-N) and the C-terminal (ZP-C) domain. However, the amino-terminal part (ZP-N) is described as a module responsible for the formation of filaments or subramolecular structures and might be responsible for protein polymerization due to disulfide bonds between cysteine residues (76, 81-83). Its conserved presence in PLAC1 is therefore likely to be important for protein–protein interactions relevant to its function, such as polymerization or interactions with heterologous protein partners.

1.4.4 PLAC1 and its role in placenta

Although the placenta specific protein PLAC1 was identified eleven years ago, only little is known about its function during human pregnancy. As mentioned above, PLAC1 mRNA expression is restricted to the placenta, especially to the cells of the trophoblast (72). Because of its restricted localization at the apical region of the maternal-facing syncytiotrophoblast, it was proposed that PLAC1 might be required for interaction between the trophoblast and other placental or maternal tissues (69, 72). Furthermore, deletion of the X chromosome region harboring the PLAC1 gene causes fetal growth restriction (runty phenotype or stillbirth) and abnormal placenta development (84-86). Since it was shown that PLAC1 expression is influenced by the keratinocyte growth factor (KGF) (72, 87), a factor that regulate normal trophoblast proliferation and differentiation, a placenta-specific PLAC1-KGF-axis regulating trophoblast development via paracrine mechanisms was hypothesized (88, 89). However, till now the molecular function of PLAC1 during placenta development is unknown.

1.4.5 PLAC1 and its role in cancer

In 2007 the placenta specific protein PLAC1 was characterized as a new tumor antigen. PLAC1 is expressed in a wide range of cancers, especially breast cancer, and in different cancer cell lines (Figure 6). Except for placenta and testis, PLAC1 is not expressed in any normal tissue (68, 73).

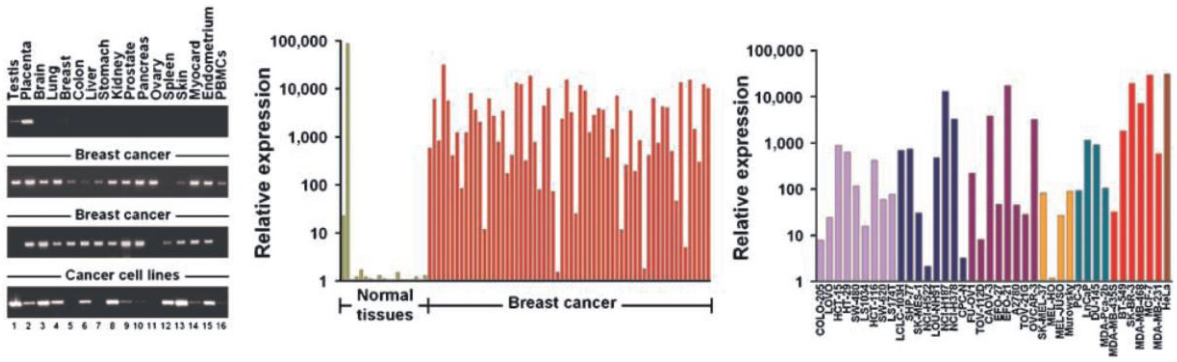

Figure 6. PLAC1 is a trophoblastic lineage marker aberrantly activated in cancer cells.
(Left) RT-PCR in normal tissues, primary breast cancer samples and cancer cell lines (1, MCF-7; 2, MDA-MB-435S; 3, BT-549; 4, MDA-MB-231; 5, SNU-16; 6, LCLC-103H; 7, KYSE-510; 8, KYSE-30; 9, EFO-27; 10, TOV-21G; 11, TOV-112D; 12, CAOV-3; 13, EFO-21; 14, FU-OV-1; 15, LNCAP; 16, CAPAN-2). (Center) Quantitative real-time RT-PCR in normal tissues (1, Testis; 2, Placenta; 3, Brain; 4, Lung; 5, Breast; 6, Colon; 7, Liver; 8; Stomach; 9, Kidney; 10, Prostate; 11, Pancreas; 12, Ovary; 13, Spleen; 14, Skin; 15, Myocard; 16, Endometrium; 17, rest. PBMCs; 18, prolif. PBMCs; 19, Adrenal gland), primary breast cancer specimens and (right) cancer cell lines (light purple, colon cancer; blue, lung cancer; dark purple, ovarian cancer; yellow, melanoma; red, breast cancer; brown, cervical cancer). (68)

The biological significance of PLAC1 in tumor cells was shown by Koslowski et al. 2007, demonstrating that cell cycle progression and consequently cell proliferation is regulated by PLAC1 in different breast cancer cell lines (Figure 7). Knockdown of PLAC1 using siRNA-induced silencing of the gene leads to an inhibition of motility, migration and invasion of breast cancer cell lines.

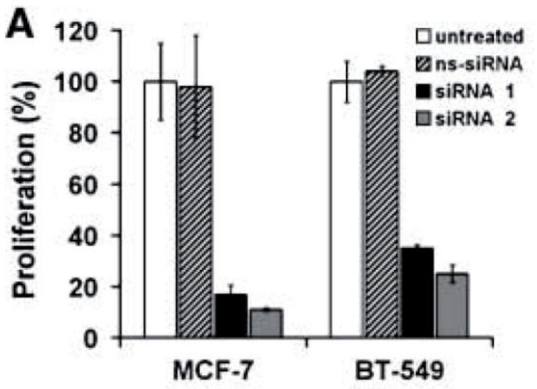

Figure 7. siRNA mediated PLAC1 knockdown inhibit proliferation.
(A) Analysis of proliferation in MCF-7 and BT-549 cells 72h after knock down has been initiated by PLAC1 specific siRNA duplexes. (68).

Furthermore, proliferation was also altered after PLAC1 knock-down leading to a G1-S arrest caused by a decreased expression of cyclin D1 and reduced phosphorylation of AKT kinase (Figure 8). But how the AKT-pathway is altered by PLAC1 remains unclear.

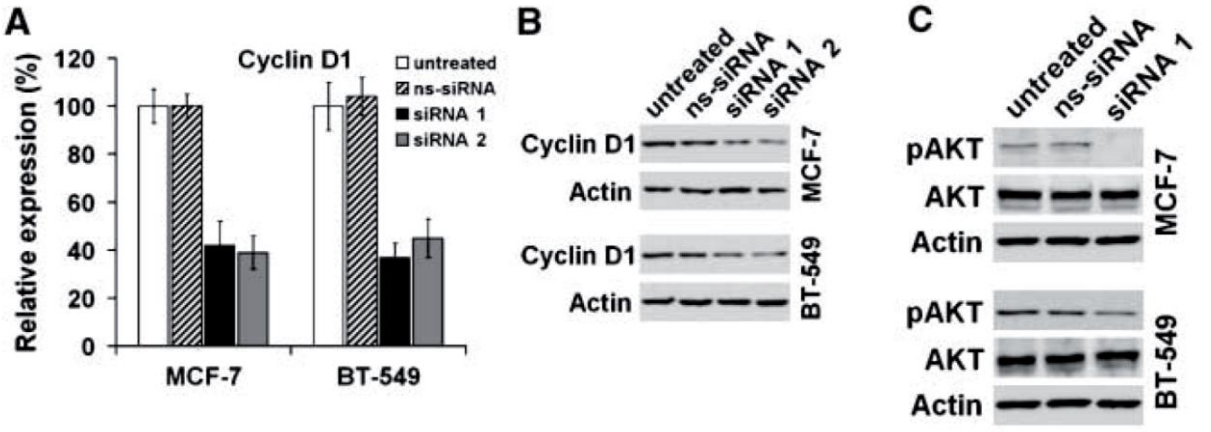

Figure 8. siRNA mediated PLAC1 knockdown inhibits proliferation and is leading to a G1-S arrest caused by a decreased expression of cyclin D1 and AKT kinase phosphorylation.
(A) Quantitative real-time RT-PCR analysis and (B) western blot analysis of cyclin D1 after cells were treated for 72h with PLAC1 specific siRNA duplexes. (C) Western blot analysis of AKT Ser473 phosphorylation after 72h of PLAC1 knockdown. (68).

1.4.6 PLAC1 gene regulation in cancer

Knowledge of the molecular and regulatory mechanism leading to the selective activation of trophoblast-specific genes in cancer is spare. However, it has been reported that the expression of several CG-genes correlate with demethylation of CpG islands in the respective promoter regions (25, 33, 66, 90, 91). In the case of PLAC1, expression is not regulated by DNA methylation (68). As previously described, the PLAC1 gene contains two different transcription start sides: promoter P1 and P2 (Figure 2). In placenta, P2 is the preferred promoter, whereas various tumor cell lines tend to express predominantly either one or the other promoter (71). The cooperation of two transcription factors, SP1 and C/EBPβ-2, are required for the expression of PLAC1 via the P2 promoter in breast cancer cells. In addition, estrogen receptor α (ERα) also seems to be involved in the regulation of PLAC1, since ERα-positive tumors display significantly higher PLAC1 expression levels compared with ERα-negative tumors. However, PLAC1 does not exhibit an estrogen-response element (ERE) in its promoter and is therefore transactivated via an indirect non-classical mechanism which

appears to be mediated by a “transcriptional cross-talk” with ligand-activated ERα binding to both transcription factors: C/EBPβ-2 and SP1 (92). For the expression of PLAC1 via the P1 promoter the cooperation of other transcription factors (RXRα in conjunction with LXRa or LXRb) are required (71).

1.4.7 PLAC1 antibody response in cancer patients

The placenta is an immunological privileged organ, which has the ability to downregulate the immune response (58). Antigens expressed in the trophoblast are unable to induce immune responses under physiological conditions. However, when these antigens are aberrantly expressed in cancer cells, specific immune responses in some cancer patients were elicit. In the case of the placenta specific protein PLAC1 only few patient exhibit a humoral immune response to PLAC1 (73, 93). This serological analysis is consistent with most CT antigens (94). Recently, a cytotoxic T-lymphocyte (CTL) epitope from PLAC1 was identified. CTLs induced by PLAC1-derived peptides were able to specifically lyze various PLAC1 expressing cell lines (95). Thus, the placenta specific protein PLAC1 can be used as target for tumor specific immune responses based on the recognition of tumor antigen by CTLs.

1.5 Purpose of the thesis

The placenta specific protein PLAC1 was proposed to be an interesting target for immunotherapeutical approaches, since it has been characterized as a cancer-testis antigen with a high expression in placenta and different cancer types and seems to be implicated in several cellular processes (e.g. proliferation, migration and invasion). However, the molecular function of PLAC1 in cancer as well as in placenta has remained elusive. The present study was carried out to characterize the placenta specific protein PLAC1 and its use for cancer immunotherapy.

To this end several cellular and biochemical approaches were carried out to address the following specific aims:

1) Analyze the protein expression of the placenta specific protein PLAC1 in different tissues and cancer cell lines.

2) Analyze the subcellular localization of the placenta specific protein PLAC1.

3) Determine PLAC1 interactions.

4) Determine the binding motives of the interaction partners.

5) Examine the molecular function of PLAC1 in placenta and in tumor cells.

II MATERIALS AND METHODS

The methods mentioned below were performed as described in Sambrook et al. (1989), Current Protocols in Molecular Biology, Current Protocols in Protein Science and Current Protocols in Cell Biology.

2.1 Material

If not indicated otherwise all chemicals and consumables were purchased from Merck (Darmstadt), Sigma Aldrich (Taufkirchen), SERVA (Heidelberg), Applichem (Darmstadt) and Roth (Karlsruhe).

2.1.1 Radioactive labelled chemicals

Radioactively labeled chemicals were purchased from Hartmann Analytic GmbH (Braunschweig):

L-[35S] Methionine: Specific activity: 37 TBq (1000Ci)/mmol

Concentration: 370 MBq (10mCi)/ml

2.1.2 Filtration and dialysis

Filtration and dialysis	
Centricon 5, 10 und 30	Sartorius AG, Göttingen, Germany
MF-Millipore membrane filter	Sigma Aldrich, Taufkirchen, Germany
Slide-A-Lyzer Dialysis Cassettes	Pierce, Rockford, USA

2.1.3 Chromatography media and chromatography columns

Chromatography columns (prepacked columns)	
HiTrap Heparin HP 1ml & 5ml	GE Healthcare, Uppsala, Sweden
HiLoad 16/60 Superdex 200 prep grade	GE Healthcare, Uppsala, Sweden
HiTrap Protein A HP 1ml & 5ml	GE Healthcare, Uppsala, Sweden

Chromatography media	
NHS-activated Sepharose 4 Fast Flow	GE Healthcare, Uppsala, Sweden
Q Sepharose Fast Flow	GE Healthcare, Uppsala, Sweden
SP Sepharose Fast Flow	GE Healthcare, Uppsala, Sweden
Glutathione Sepharose 4 Fast Flow	GE Healthcare, Uppsala, Sweden
Nickel-NTA Superflow	Qiagen, Hilden, Germany
Protein A Sepharose Fast Flow	Sigma Aldrich, Taufkirchen, Germany
Protein G Sepharose Fast Flow	Sigma Aldrich, Taufkirchen, Germany
Heparin Agarose Fast Flow	Sigma Aldrich, Taufkirchen, Germany

2.1.4 Molecular weight standards

High and low molecular weight standards for size exclusion chromatography	
Carbonic anhydrase, from bovine erythrocytes, 29,000 MW	Sigma Aldrich, Taufkirchen, Germany
Aprotinin, from bovine lung, 65,00 MW	Sigma Aldrich, Taufkirchen, Germany
Albumin, from bovine serum, 66,000 MW	Sigma Aldrich, Taufkirchen, Germany
Alcohol dehydrogenase, from yeast, 150,000 MW	Sigma Aldrich, Taufkirchen, Germany
β-Amylase, from sweet potato, 200,000 MW	Sigma Aldrich, Taufkirchen, Germany
Apoferritin, from horse spleen, 443,000 MW	Sigma Aldrich, Taufkirchen, Germany
Thyroglobulin, from bovine, 669,000 MW	Sigma Aldrich, Taufkirchen, Germany

Protein Standards	
BenchMark	Invitrogen, San Diego, USA
SeeBlue Plus 2	Invitrogen, San Diego, USA
MagicMark XP	Invitrogen, San Diego, USA

DNA Standards	
GeneRuler 50bp Ladder	Fermentas, Burlington, USA
GeneRuler 1kb Ladder	Fermentas, Burlington, USA

2.1.5 Complex reagents and kits for molecular biology

Complex reagents	
Complete without EDTA, protease inhibitor cocktail	Roche, Mannheim, Germany
Lipofectamine 2000, transfection reagent	Invitrogen, San Diego, USA
Lipofectamine LTX and PLUS, transfection reagent	Invitrogen, San Diego, USA
Rotiphorese Gel 30	Roth, Karlsruhe, Germany
Lumi-light western blotting substrate	Roche, Mannheim, Germany
Super signal west dura maximum sensitivity substrate	Pierce, Rockford, USA
Super signal west femto maximum sensitivity substrate	Pierce, Rockford, USA
TNT-SP6/T7 coupled reticulocyte lysate system	Promega, Mannheim, Germany
Desoxynukleotid-set	Sigma Aldrich, Taufkirchen, Germany

Kits for molecular biology	
BCA protein assay reagent kit	Pierce, Rockford, USA
NucleoBond plasmid kits (Mini, Midi, Maxi)	Macherey-Nagel, Düren, Germany
Qiaquick gel extraction kit	Qiagen, Hilden, Germany
Quick change site-directed mutagenesis kits	Agilent Technologies, Santa Clara, USA

2.1.6 Buffers and solutions

name	*composition*
Amido black staining solution	0.2% (w/v) Amido black 10B 10% (v/v) methanol 2% (v/v) acetic acid
Blocking buffer	50mM Tris/HCl 5.2M NaCl 0,01% (w/v) Triton X-100, pH 7.0
Coomassie- staining solution	50% (v/v) methanol 10% (v/v) acetic acid 0.1% (w/v) Coomassie Billant Blue
Co-immunoprecipitation- lysis buffer	1x PBS 0.01% (w/v) Triton X-100 2mM EDTA 2mM EGTA 5% (v/v) β-Mercaptoethanol protease inhibitor cocktail, pH 7.5

Co-immunoprecipitation- wash buffer	1x PBS 0.01% (w/v) Triton X-100 2mM EDTA 2mM EGTA 5% (v/v) β-Mercaptoethanol 0.1% (w/v) BSA protease inhibitor cocktail, pH 7.5
GST- purification buffer	300mM NaCl 50mM Tris-HCl 2.5mM EDTA 2.5mM EGTA 0,01% Triton X-100 1mM DTT protease inhibitor cocktail, pH 7.5
Glutathione buffer	0.03% (w/v) glutathione in GST-purification buffer
Na-tetraborate buffer	0.2M Na-Tetraborate, pH 9.0
PBS	140mM NaCl 2.7mM KCl 10mM Na_2HPO_4 1.8mM KH_2PO_4
PBS-T	0.05% (w/v) Tween-20 in PBS
SDS electrophoresis buffer	25mM Tris 192mM Glycerin 0.1% (w/v) SDS
SDS lysis buffer, 4x	250mM Tris/HCl 34% (w/v) Glycerin 8.2% (w/v) SDS 5 % (v/v) β-Mercaptoethanol, pH 6.8
SDS sample buffer, 10 x	209mM Tris/HCl 41% (w/v) Glycerin 7.7% (w/v) SDS 0.003% (w/v) Bromophenol blue 17% (v/v) β-Mercaptoethanol, pH 6.8
SDS-stacking gel solutions, 4 x	0.4% (w/v) SDS 0.5M Tris/HCl, pH 6.8
SDS-separating gel solutions, 4 x	0.4% (w/v) SDS 1.5M Tris/HCl, pH 8.8

Transfer buffer	25mM Tris 192mM Glycine 20% (v/v) methanol
Fix-solution	50% (v/v) methanol 12% (v/v) acetic acid 0.0185% (w/v) Formaldehyde
Silver stain solution	0.2 % (w/v) Silver nitrate 0.028% (w/v) Formaldehyde
Developing solution	6% (w/v) Natriumcarbonat 0.0185% (w/v) Formaldehyde
Stop-solution	50% (v/v) methanol 12% (v/v) acetic acid
Protein A-purification equilibration buffer	1M Monosodium phosphate 1M Disodium hydrogen phosphate, pH7.0
Protein A-purification elution buffer	0.1M Glycine M Tris/HCl, pH 3.0
FGF7-purification equilibration buffer	0.2M NaCl M Tris/HCl, pH 7.5
FGF7-purification elution buffer	2.0M NaCl, M Tris/HCl, pH 7.5
Size exclusion chromatography buffer	150mM NaCl 50mM Tris 1mM DTT, pH 7.5
TAE-buffer	40mM Tris-Acetate, pH 8.0 1mM EDTA

2.1.7 Cell culture media

name	
DMEM	Invitrogen, San Diego, USA
F12	Invitrogen, San Diego, USA
DMEM-F12	Invitrogen, San Diego, USA
RPMI	Invitrogen, San Diego, USA
MEM	Invitrogen, San Diego, USA
Pro-293a-CDM	Lonza, Cologne, Germany

2.1.8 Bacteriological media

name	*composition*
LB-medium	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl
Super broth	3.5% (w/v) tryptone, 2% (w/v) yeast extract, 0.5% (w/v) NaCl
SOC-medium	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 20mM Glucose

To obtain solid media 1.5% (w/v) agar was added to the bacteriological media described before. Depending on the selection markers 100µg/ml ampicillin or 50µg/ml kanamycin were added to the media.

2.1.9 Antibodies

primary antibodies	
Goat anti-FGF7 (AF-251-NA)	R&D-Systems, Wiesbaden, Germany
Goat anti-FGF7 (K-4760)	Sigma Aldrich, Taufkirchen, Germany
Mouse anti-beta Actin (A1978)	Sigma Aldrich, Taufkirchen, Germany
Mouse anti-c-Myc (M4439)	Sigma Aldrich, Taufkirchen, Germany
Mouse anti-HA.11 (raw ascites fluid)	Covance, New Jersey, USA
Rabbit anti-GFP FL3 (DB088)	Delta Biolabs, Gilroy, USA
Rabbit anti-c-Myc (A-14) sc-789	Santa Cruz Biotechnology, Delaware, USA
Mouse anti-Stat3 (124H6)	Cell Signaling, Boston, USA
Mouse anti-Lamin (65147)	Progen, Heidelberg, Germany
Mouse anti-Calreticulin (FMC75)	Abcam, Cambridge, United Kingdom
Mouse anti-PLAC1 (78H11-1H6)	Ganymed AG, Mainz, Germany
Mouse anti-PLAC1 (22-3A-1.4)	Ganymed AG, Mainz, Germany
Rabbit anti-PLAC1 (Serum11)	Tulip AG, Mainz, Germany
Mouse anti-PLAC1 (23-32-A1.2)	Ganymed AG, Mainz, Germany

secondary antibodies	
Rabbit Anti-Mouse IgG (Fc-specific), HRPO (31455)	Pierce, Rockford, USA
Goat Anti-Rabbit IgG (Fc-specific), HRPO (31463)	Pierce, Rockford, USA
Rabbit Anti-Goat IgG (Fc), HRPO (31433)	Pierce, Rockford, USA
Goat Anti-Mouse IgG (H+L) [F(ab')2], HRPO (31438)	Pierce, Rockford, USA
Goat anti-Mouse IgG (H+L), CY3 (115-165-0063)	Jackson, Suffolk, UK
Goat anti-Mouse IgG (H+L), CY2 (115-225-003)	Jackson, Suffolk, UK

2.1.10 Enzymes

Enzymes were purchased from New England Biolabs (Schwalbach, Germany) and MBI Fermentas (Burlington, USA). Unless not otherwise specified, reactions were performed as described by the manufacturers.

2.1.11 Oligonucleotide sequences

DNA- Oligonucleotide sequences were purchased at MWG-Biotech (Ebersberg, Germany).

Oligonucleotides for cloning

plasmid name	*oligonucleotide sequences*
pCR3.1-hPLAC1 1-212aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-197aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC-3´
	5´- TATCTCGAGGGATGTCCAGGAAGTGGCTGGG-3´
pCR3.1-hPLAC1 1-182aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC-3´
	5´- TATCTCGAGGTCCGGCCTGGTGGCAGGGCAC-3´
pCR3.1-hPLAC1 1-167aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC-3´
	5´- TATCTCGAGGCACGCAGGGGGGGCAGTCGCA-3´
pCR3.1-hPLAC1 1-152aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC-3´
	5´- TATCTCGAGGCAGGCTGAACACCTCGTAGCA -3´
pCR3.1-hPLAC1 1-137aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC-3´
	5´- TATCTCGAGGCCGGCTCTTGCTGGCCACCCG-3´
pCR3.1-hPLAC1 1-122aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC-3´
	5´-TTAATCTCGAGGGCTCTTCTGAGGGGCGGCACA-3´
pCR3.1-hPLAC1 1-107aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´
	5´- TATCTCGAGGGCTGGGGGTGCCCTTGCTGCT-3´
pCR3.1-hPLAC1 1-92aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´
	5´- TATCTCGAGGCACCATGTCCTGGCTCACGGC-3´
pCR3.1-hPLAC1 1-77aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´

	5´- TATCTCGAGGCACCCGGTAGGTGAACTGGTA-3´
pCR3.1-hPLAC1 1-24aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´
	5´-TATGCGGCCGCGCTCTGGCCGCT-3´
pCR3.1-hPLAC1 1-24+25-212aa myc	5´-ATAGCGGCCGCACCCATGACCGTGCTGTGCAGC-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-24+33-212aa myc	5´-ATAGCGGCCGCAGACTGGTTCATGGTGACC-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-24+48-212aa myc	5´-ATAGCGGCCGCAGTGTGCGTGCACTTCCACG-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-24+63-212aa myc	5´-ATAGCGGCCGCACCCAACCACGTGCAGCCC-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-24+76-212aa myc	5´-ATAGCGGCCGCAACCGAGTGCGGCATCC-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-24+93-212aa myc	5´-ATAGCGGCCGCAATCTACAGCACCGAGATCC-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-24+108-212aa myc	5´-ATAGCGGCCGCAAAGTTCGTGATCCCCGTG-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-24+116-212aa myc	5´- GCGGCCGCCGCCCCTCAGAA-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 23-212aa myc	5´-ATAGAATTCGCCACCATGCAGAGCCCCATGACC-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 1-122aa myc	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´
	5´-TTAATCTCGAGGGCTCTTCTGAGGGGCGGCACA-3´
pCR3.1-hPLAC1 116-212aa myc	5´ATAGAATTCGCCACCATGGCCGCCCCTCAGAAGAGC-3´
	5´-TATCTCGAGGCATGCTGCCGATCATGTCGTCGGT-3´
pCR3.1-hPLAC1 25-122aa myc	5´-ATAGAATTCGCCACCATGCAGAGCCCCATGACC-3´
	5´-TTAATCTCGAGGGCTCTTCTGAGGGGCGGCACA-3´
pCR3.1-hPLAC1 1-212aa	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´
	5´-GAGCTCGAGTCACATGCTGCCGATCATGTCGTC-3´
pCR3.1-hPLAC1 23-212aa	5´-ATAGAATTCGCCACCATGCAGAGCCCCATGACC-3´
	5´-GAGCTCGAGTCACATGCTGCCGATCATGTCGTC-3´
pCR3.1-hPLAC1 1-122aa	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´
	5´-GAGCTCGAGTCAGCTCTTCTGAGGGGCGGCACA-3´
pCR3.1-hPLAC1 25-122aa	5´- ATAGAATTCGCCACCATGAAGGTGTTCAAGTTC -3´
	5´-GAGCTCGAGTCAGCTCTTCTGAGGGGCGGCACA-3´
pCR3.1-hPLAC1 116-212aa	5´- GCGGCCGCCGCCCCTCAGAA-3´
	5´-GAGCTCGAGTCACATGCTGCCGATCATGTCGTC-3´
pCR3.1-hPLAC1 1-24+116-212aa	5´- GCGGCCGCCGCCCCTCAGAA-3´
	5´-GAGCTCGAGTCACATGCTGCCGATCATGTCGTC-3´
pSEC-hPLAC1 23-212aa	5´-CTCGCTAGCCAGAGCCCCATGACCGTGC-3´
	5´-GAGGGATCCTCACATGCTGCCGATCATG-3´

pCR3.1-eGFPmyc	5´-ATAGAATTCGCCACCATGGTGAGCAAGGGC-3´ 5´-TATCTCGAGTCTTGTACAGCTCGTCCATG-3´

Oligonucleotides for RT-PCR

name	*oligonucleotide sequences*
PLAC1_for	5´-AAA TTT GGC AGC TGC CTT CAC-3´
PLAC1_rev	5´-TGA TGC CAC ATT CAG TAA CAC-3´
HPRT_for	5´-TGA CAC TGG CAA AAC AAT GCA-3`
HPRT_rev	5`-GGTCCT TTT CAC CAG CAA GCT-3´

2.1.12 Mammalian and bacterial protein expression vectors

mammalian expression vectors	
pCR3.1	Invitrogen, Groningen, Netherlands
pcDNA3.1	Invitrogen, Groningen, Netherlands
pEGFP-C1	Invitrogen, Groningen, Netherlands
pFUSE-rFc2 (IL2ss)	InVivoGen, San Diego, USA

bacterial expression vectors	
pGEX-6P.1	GE Healthcare, Uppsala, Sweden
pET-32a	Merk, Darmstadt, Germany

2.1.13 Bacterial stains and eukaryotic cell lines

bacterial stains	
BL21(DE3)	F– *omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3)
XL1blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lac*Iq*ZΔM15* Tn*10* (Tetr)]
Top10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
DH5α	F– Φ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1

eukaryotic cell lines	
HEK293	human embryonic kidney cancer cell line
SKBR-3	human mammary gland adenocarcinoma cell line
MCF7	human mammary gland adenocarcinoma cell line
BT-20	human mammary gland carcinoma cell line
JIMT-1	human mammary gland carcinoma cell line
MDA-MB-231	human mammary gland adenocarcinoma cell line
MDA-MB-435S	human mammary gland carcinoma cell line
MDA-MB-361	human mammary gland adenocarcinoma cell line derived from metastatic brain
NUGC-4	human gastric carcinoma cell line
JAR	human placental choriocarcinoma cell line
BeWo	human placental choriocarcinoma cell line
JEG-3	human placental choriocarcinoma cell line
LA1-5s	human neuroblastoma cell line
LA-55n	human neuroblastoma cell line
SH-SY5Y	human brain neuroblastoma cell line derived from metastatic bone marrow
Kelly	human liver hepatocellular carcinoma cell line
IMR-32	human neuroblastoma cell line derived from metastatic abdominal mass
SK-N-F1	human neuroblastoma cell line derived from metastatic bone marrow
SK-N-DZ	human neuroblastoma cell line derived from metastatic bone marrow
NEC-8	human embryonal testicular carcinoma cell line
MelHo	human melanoma cell line
AGS	human gastric adenocarcinoma cell line
KATOIII	human gastric carcinoma cell line derived from metastatic axillary lymph nodes
BxPC-3	human pancreatic adenocarcinoma cell line
CAPAN-1	human pancreatic adenocarcinoma cell line derived from metastatic liver
KP-2	human pancreatic cancer cell lines
KP-4	human pancreatic ductal cell carcinoma cell line

KCI-MOH	human pancreatic cancer cell line
HPAC	human pancreatic adenocarcinoma cell line
Panc02.03	human pancreatic adenocarcinoma cell line
Panc04.03	human pancreatic adenocarcinoma cell line
Panc05.04	human pancreatic adenocarcinoma cell line
SK-OV-3	human ovary adenocarcinoma cell line derived from metastatic ascites
PA-1	human ovary teratocarcinoma cell line derived from metastatic ascites
ES-2	human ovary carcinoma cell line
DU145	human prostate carcinoma cell line derived from metastatic ascites brain
NCI-H929	human myeloma cell line
HeLa	human cervix adenocarcinoma cell line
HEK293T	human embryonic kidney cancer cell line 293 transformed with adenovirus and expressing the SV40 T-antigen

All cell lines were purchase from the ATCC or DSMZ and were cultured as recommended by the provider.

2.1.14 RNAi

RNAi - duplexes sequences		
PLAC1 siRNA (1)	5´-CUCCAUGAGAGUAGCCAGCAA-3´	Qiagen, Hilden, Germany
PLAC1 siRNA (4)	5´-CCGGUUCAGGACAAAGTCCAA-3´	Qiagen, Hilden, Germany
ns RNA (control)	5´-UAACUGUAUAAUCGACUAG-5´	Thermo, Schwerte, Germany

2.2 Methods

Methods in molecular biology

2.2.1 Polymerase chain reactions

For the enzymatic amplification of DNA (PCR) the High Fidelity DNA Polymerase (Finnzymes, Finland) was used. A typical PCR mix consist of template DNA (1pg-10ng), the appropriate oligonucleotide primers (final conc. each 5µM), deoxyribonucleoside triphosphates (dNTPs, final conc. each 2mM), 0.02 U/µl DNA polymerase, buffer and water. Once assembled, the mixture is subjected to a standard cycling procedure. The first step is an initial DNA denaturation, after that many repeating cycles were performed (usually 30 times), permitting sequential steps of DNA denaturation, primer annealing and DNA synthesis, to exponentially amplify a product of specific size and sequence. The PCR products were subsequently displayed on an appropriate gel and examined for yield and specificity.

2.2.2 Digestion of cDNA with restriction endonucleases

Restriction endonucleases are able to cleave double-stranded cDNA at specific recognition nucleotide sequences known as restriction sites. A directed cleavage of cDNA is also called digestion. Restriction digests were performed according to the manufacturer´s protocol (MBI Fermentas, Burlington, USA).

2.2.3 Dephosphorylation of cDNA

Alkaline Phosphatase catalyzes the release of 5'- and 3'-phosphate groups from cDNA. Dephosphorylation of cDNA is used to prevent recircularization of cloning vector cDNA during ligation. Dephosphorylation was performed according to the manufacturer´s protocol (MBI Fermentas, Burlington, USA).

2.2.4 Agarose gel electrophoresis

To separate and purify cDNA fragments agarose gel electrophoresis was performed. There for electrophoresis-grade agarose, electrophoresis buffer (1x TAE-buffer) and ethidium bromide were used. The appropriate agarose concentration for separating DNA fragments of various sizes was adjusted. cDNA samples were mixed with an assigned amount of 6x DNA-loading buffer. The proper DNA molecular weight marker and cDNA-samples were loaded on the

agarose gel and electrophoresis was performed at 3V/cm. When the bromophenol blue dye from the loading buffer has migrated a distance judged sufficient for separation of the cDNA fragments, the gel was documented on a UV transilluminator.

2.2.5 cDNA extraction and purification from TAE-agarose gels

The extraction and purification of cDNA fragments from agarose gels was performed with the QIAquick Gel Extraction Kit according to the manufacturer´s protocol (Qiagen, Hilden, Germany). This method is based on solubilization of agarose and selective, quantitative adsorption of nucleic acids to the QIAEX II silica-gel particles in the presence of high salt. Elution of the cDNA is accomplished with a low-salt solution such as Tris buffer or water.

2.2.6 Ligation of DNA fragments

DNA ligation is used to insert a cDNA fragment into a plasmid. The T4 DNA ligase catalyzes the formation of phosphodiester bonds between 5'-phosphate and 3'-hydroxyl termini. Ligation was performed using T4-DNA-Ligase according to the manufacturer´s protocol (MBI Fermentas, Burlington, USA).

2.2.7 Site-directed mutagenesis

Point mutations, replacement of amino acids and deletion or insertions of single or multiple adjacent amino acids were generated by so called site directed mutagenesis.

The PCR based mutagenesis of plasmid DNA was performed using the „Quick change site-directed mutagenesis kits" according to the manufacturer´s protocol (Agilent Technologies, Santa Clara, USA).

2.2.8 Transformation of competent *E. coli* cells

For plasmid DNA amplification or recombinant protein expression, plasmids were transformed into appropriate chemo-competent *E. coli* strains (purchased from Invitrogen, Netherlands) by heat shock. Thereto competent cells were incubated on ice for 30 minutes with 0.1-50ng DNA followed by a heat-pulse at 42°C for 45 seconds. Preheated SOC medium was added to the transformed bacteria and incubated for 1 hour at 37°C and 225-250 rpm. Transformed *E. coli* were grown overnight at 37°C on LB agar plates containing ampicillin or kanamycin for selection, depending on the resistance expressed by the transformed plasmid.

2.2.9 Plasmid DNA purification

Purification of plasmid DNA was performed with a silica-based anion-exchange resin, developed by Macherey-Nagel. It consists of hydrophilic silica beads functionalized with MAE (methyl-amino-ethanol). The dense coating of this functional group provides a high overall positive charge density that permits the negatively charged phosphate backbone of plasmid DNA to bind. All contaminants from proteins to RNA are washed from the column. The positive charge of the resin is neutralized by a pH and pure plasmid DNA is eluted in a high-salt elution buffer. DNA purification was performed according to the manufacturer´s protocol (Macherey-Nagel).

2.2.10 Spectrophotometric analysis of nucleic acids

Spectrophotometry was performed to determine the DNA or RNA concentrations and purity. In a spectrophotometer, a sample is analyzed by ultraviolet light at a wavelength of 260nm. Using the Beer Lambert Law it is possible to relate the amount of light absorbed to the concentration of the DNA concentration. An optical density of 1 corresponds to a concentration of 50µg/ml for double stranded DNA. The ratio of the absorbance at 260 and 280nm ($A_{260/280}$) is used to assess the purity of nucleic acids (DNA: $A_{260/280}$ is ~1.8).

2.2.11 Sequencing of DNA

Purified DNA was sequenced by a commercial provider (MWG Eurofins, Ebersberg, Germany) with appropriate sequencing primers to determine the presence and orientation of the insert.

2.2.12 RNA isolation

Purification of total RNA from eukaryotic cells was performed with RNeasy Mini Kit (Qiagen) according to the manufacturer´s protocol. Up to $1x10^7$ cells were lyzed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which inactivates RNases to ensure purification of intact RNA. Prior to loading the sample into an RNeasy Mini spin column, ethanol was added to provide appropriate binding conditions. Total RNA binds to the silica-membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30-50µl water. Finally, spectrophotometry was performed to determine the RNA concentrations and purity.

2.2.13 Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR). In RT-PCR, an RNA strand is first reverse transcribed into its DNA complement (complementary DNA or cDNA) using the enzyme reverse transcriptase. A typical RT-PCR mix consist of template RNA (10ng), the oligo-dT primer (final conc. 10µM), deoxyribonucleoside triphosphates (dNTPs, final conc. each 2mM), 200U reverse transcriptase, buffer and water. Once assembled, the reaction takes place at 42°C and is then inactivated at 70°C. Finally, quality of cDNA synthesis was checked by performing a standard PCR amplifying housekeeping gen e.g. p53, using the new synthesized cDNA as template.

2.2.14 Real-time reverse transcription-PCR

Real-time polymerase chain reaction or quantitative real time polymerase chain reaction (qPCR) is used to amplify and simultaneously quantify a targeted DNA molecule. The key feature of qPCR is that the amplified DNA is detected as the reaction progresses in real time using a non-specific fluorescent dye (SYBR Green, Qiagen) that intercalate with any double-stranded DNA. Real-time quantitative expression analysis was done in triplicates in a 40-cycle RT-PCR. For normalization the housekeeping gen hypoxanthine phosphoribosyl transferase (HPTR) was used. PLAC1 transcripts of siRNA transfected cells were quantified relative to nsRNA transfected cells using ΔΔCT calculation.

Methods in cell biology

2.2.15 Cultivation of eukaryotic cells

Depending on the cell line an appropriate basic medium such as Eagle minimal essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), Dulbecco's modified Eagle medium F12 (DMEM+F12), F12 Eagle medium (F12), and RPMI 1640 was applied. A basic medium is supplemented by addition of antibiotics (1% (v/v) penicillin and streptomycin sulfate) and usually fetal bovine serum (10%) to formulate a "complete medium".

2.2.16 Determination of eukaryotic cell density by a Neubauer chamber

To determine the density and vitality of a cell suspension a hemacytometer (Neubauer chamber) in combination with trypan blue was used. A hemacytometer is a thick glass slide with a central area designed as a counting chamber. Cell suspension is applied to a defined

area and counted so that overall cell density can be calculated. Cell vitality was addressed using typhan blue, a vital diazo dye that selectively stains dead cells blue. Live cells with intact cell membranes are not stained.

2.2.17 Freezing / thawing of eukaryotic cells

It is sometimes necessary to store cell lines for future studies; there for cell lines may be frozen for long-term storage. Generally, a cryoprotective agent such as dimethylsulfoxide (DMSO) is used for preserving cells at -80◦C or lower. DMSO acts to reduce the freezing point and allows a slower cooling rate. Gradual freezing reduces the risk of ice crystal formation and cell damage. For cryopreservation cells were cultured in logarithm-phase (log-phase) growth. Adherent cells were trypsinized, washed with cold PBS (4°C), counted in Neubauer chamber and centrifuged (300xg, 4°C, 8min). After removal of the supernatant, the pellet was resuspended in a required volume of cold freezing medium (4°C) to obtain a cell density of 1 2x107 cells/ml. Further the cell suspension (1ml aliquots) was transferred into labeled cryo-vials, placed overnight in a -80◦C freezer and then transfer to a liquid nitrogen storage freezer for long term storage.

For thawing, cells in the cryo-vial were incubated in a water bath (37°C) until the cell suspension was completely thawed. Cells were washed with complete medium and centrifuged (300xg, 4°C, 8min) to get rid of DMSO. After removal of the supernatant, the pellet was resuspended in complete medium and cells were plated at high density to optimize recovery.

2.2.18 Transient transfection of eukaryotic cells with cDNA

To transfect DNA into mammalian cells Polyethylenimine (PEI) supplied by Polysciences (United Kingdom) was used. One day before transfection, adherent cells (e.g. HEK293T) were plated in complete medium without antibiotics so that cells obtained 90-95% confluence at the time of transfection. The amounts of PEI, DNA as well as platting medium were adjusted to each culture recipient:

Culture vessel	Surf. Area per well	Vol. of platting medium	Vol. of dilution medium	cDNA	PEI (stock solution: 1mg/ml)
6-well	10 cm^2	2 ml	0,25 ml	1,5 µg	10 µg
6 cm	28,7 cm^2	5 ml	0,5 ml	8 µg	30 µl
10 cm	78,5 cm^2	15 ml	1 ml	24 µg	80 µl
13,5 cm	143,2 cm^2	30 ml	1,5 ml	45 µg	150 µl

Before transfection, platting medium was changed using the basic medium without serum. On the other side an appropriate amount of cDNA and PEI was diluted in medium and incubated at room temperature for 30 minutes. Afterwards, the mixture of cDNA/PEI was added drop wise to the plates and incubated at 37°C in a CO_2 incubator for 48 hours. Medium was changed 8 hours post-transfection (complete medium with antibiotics) to avoid apoptotic effects of PEI.

For gene expression studies in cell lines that are difficult to transfect (e.g. BeWo, MCF7, HeLa) Lipofectamine LTX Plus (Invitrogen, Germany) was applied following the manufacturer's instructions.

2.2.19 Transient transfection of eukaryotic cells with small interfering RNA

The application of RNA interference (RNAi) to mammalian cells has the ability to simply, effectively, and specifically downregulate the expression of genes. Efficient transfection of siRNA is critical for gene silencing. For PLAC1 silencing studies, cells were transfected with 10nM siRNA duplexes using the HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. For the gen silencing experiments performed within this study a mixture of two different siRNA duplexes (Qiagen) targeting PLAC1 mRNA sequence (siRNA 1 targeting nucleotides 342 to 362 and siRNA 4 targeting nucleotides 670 to 690) and as non-silencing control RNA, a scrambled siRNA duplex, were used.

2.2.20 Cell proliferation analysis

Proliferation was measured using the Delfia Cell Proliferation Kit. The Delfia Cell Proliferation assay is a fluoroimmunoassay based on the incorporation of Bromodeoxyuridin (BrdU), a synthetic nucleoside that is an analogue of thymidine, into newly synthesized DNA of proliferating cells. Incorporated BrdU is detected using a europium labeled monoclonal antibody. In a final step an inducer is added to form highly fluorescent chelates with the dissociated europium ions from the labeled antibody. The fluorescence measured in the cell population is proportional to the DNA synthesis of the cells. 24h after transfection with siRNA duplexes, $5x10^3$ cells were cultured for 24h, 48h and 72h in 96-well plates in medium supplemented with 10% FCS or 2% FCS and finally proliferation was analyzed according to the manufacturer's instructions on a Wallac Victor2 multi-label counter (Perkin-Elmer).

Methods in biochemistry

2.2.21 Generation of protein extracts

Two different kinds of generation of protein extracts from tissue culture cells were performed in this work: Whole denatured protein extracts and soluble native protein extracts.

To generate whole denatured protein extracts, cells were washed once with PBS and subsequently scraped from the cell culture plate applying a 4x SDS- lysis buffer. Finally, the sample was subjected to sonication.

For the generation of soluble native protein extracts, cells were carefully washed by pipetting from the cell culture plate in PBS and pelleted by centrifugation (8min, 1200rpm, 4°C). Pellet was resuspended in lysis buffer and cells were broken by sonication. Subsequently a high-speed centrifugation (21000g, 30min, and 4°C) was performed.

2.2.22 Quantification of the protein concentration

Protein concentration can be determined by many methods. To estimate the protein concentration in an extract, protein absorbance at 280nm was measured (OD_{280}=1, corresponds to 1mg/ml protein concentration). Moreover, concentration of purified recombinant proteins has also been determined by SDS-PAGE followed by Coomassie blue staining and quantification using an accurately defined protein as referents. In some cases protein concentration was also determined by a "BCA protein assay kit" (Pierce, USA) according to the manufacturer´s protocol.

2.2.23 Discontinuous gel electrophoresis (SDS-PAGE)

One-dimensional gel electrophoresis separates proteins based on their molecular size as they move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is casted as a separating gel and topped by a stacking gel. The desired percentage of acrylamide in the separating gel depends on the molecular size of the proteins being separated. Generally, 5% gels are used for SDS-denatured proteins of a size of 60 to 200 kDa, 10% gels for SDS-denatured proteins of a size of 16 to 70 kDa, and 15% gels for SDS-denatured proteins of 12 to 60kDa in size. After sample proteins are solubilized and denatured by boiling at 98°C for 5 minutes in the presence of SDS, an aliquot of the protein solution is applied to a gel lane, and the individual proteins are separated electrophoretically. β-Mercaptoethanol is added during boiling to reduce disulfide bonds.

2.2.24 Protein detection by Coomassie blue staining

To detect the separated proteins of a polyacrylamide gel, staining with a Coomassie brilliant blue staining solution was performed. Coomassie brilliant blue binds non-specifically to almost all proteins. The SDS-gel was incubated for 15min with Coomassie solution and subsequently incubated with a destaining solution. Destaining eliminates the background while the protein bands retain the blue colour. Gels were digitally documented by scanning and in some cases sealed in a plastic bag with a small amount of destaining solution and stored for several months at 4°C.

2.2.25 Protein detection by silver staining

Silver staining is a very sensitive method to stain proteins (0.1ng). To prevent protein diffusion and reduce background, gel was fixed after electrophoresis by incubating it for at least 3hr or overnight in fixation solution. To remove fixing solution the gel was washed three times each 20min with a 50% (v/v) ethanol solution. This step was follow-up with 1min incubation with 0.02% (w/v) sodium thiosulfate solution, further by a wash step with water (3x 20s) and finally incubated with a silver stain solution. To remove the silver nitrate solution, repeating washing step with water were performed. Developing solution was added till brown precipitates were visualized (30 sec) and was removed quickly with water to prevent background. The reaction was stopped adding stop-solution.

2.2.26 Autoradiography

To visualize radio-labeled proteins (^{35}S) after electrophoresis and to reduce background of free, unbound radioactivity, SDS-Pages were stained with Coomassie as described in 3.4.1.3. Gels were vacuum-dried at 80°C for 20 minutes and exposed to an X-ray film at -80°C. To obtain greater sensitivity a Kodak BioMax TransScreen LE was applied.

Immunological und immunbiochemical methods

2.2.27 Immunoblotting: Electroblotting of proteins onto retentive membranes and immunodetection of proteins with specific antibodies (Western blot)

Proteins separated on polyacrylamide gels were blotted onto 0.1µm nitrocellulose membranes via the "semi-dry" or "wet" electroblotting method.

For "semi-dry"-electroblotting, six sheets of filter paper, the transfer membrane and the gel containing proteins of interest were equilibrated in transfer buffer. The transfer sandwich consist of three sheets of filter paper placed on the anode of the transfer unit followed by the nitrocellulose membrane, the gel and three more sheets filter paper. Finally the cathode was attached on top of the transfer sandwich and proteins were transferred for 1,5h at 1,5mA/cm2 of gel surface area.

For electroblotting with a tank transfer unit ("wet"-blotting) four sheets of filter paper, the transfer membrane and the gel containing proteins of interest were equilibrated in transfer buffer. The transfer sandwich consists of two sheets of filter paper followed by the nitrocellulose membrane, the gel and two more sheets filter paper. This sandwich was trapped between two fiber pads in a plastic gel holder cassette. The assembled cassette is then placed in a tank containing transfer buffer. Protein transfer was performed with the Bio-Rad Trans-Blot tank transfer apparatus with plate electrodes (at 200mA for 2h).

To proof that the protein transfer was successful, membranes were incubated with an amido black -staining solution for a few minutes and destained with PBS.

For protein detection membranes were incubated with specific antibodies. To block unspecific binding of the antibody, membranes were incubated with 5x blocking buffer for at least 1h at room temperature. Afterwards the membrane was incubated with primary antibody diluted in 1x blocking solution over night at 4°C. After tree wash cycles each for 10min in PBS-T, the membrane was incubated for 1h with a specific secondary horseradish peroxidase (HRPO) -conjugated antibody diluted in blocking solution. Finally the membrane was washed three times each 10 min with PBS-T.

After the incubation with the primary and secondary antibody, bound antigens were visualized by chemo luminescence. Therefore membranes were incubated for 2min in an ECL-substrate solution and subsequently protein bands were detected with the Luminescent Image Analyzer LAS-3000.

In the case of very weak signals an ultra-sensitive enhanced chemiluminescent substrate (SuperSignal West Femto Substrate, Pierce) was applied to enhance sensitivity.

2.2.28 Detection of protein-protein interactions by co-immunoprecipitation

Co-precipitation of proteins from whole cell extracts is a valuable approach to analyze interactions between proteins *in vivo* or *in vitro*.

Co-immunoprecipitations were performed at 4°C with precooled buffers. The specific antibody (5µg) was coupled to previously activated NHS-sepharose or protein-A-sepharose (20µl) and cross-linked to the matrix with Dimethylpimelimidate (DMP).

DMP is a water soluble homobifunctional imidoester cross linker. Antibody coupled beads were equilibrated two times with a Na-tetraborate buffer, pH 9.0 and cross linked with 0.052g/10ml DMP for 30min at room temperature on a rotating wheel. Reaction was stopped with blocking buffer.

To analyze protein-protein interactions, cells expressing the desired proteins were lyzed in IP-lyses-buffer by sonication and centrifuged (30min, 21000g, 4°C) to pellet all cell debris. The supernatant was transferred to a new tube. To get rid of all unspecific binding proteins a preclearing step with empty sepharose/agarose matrix for 1h at 4°C on a rotating wheel was performed. To pellet the preclearing beads and all nonspecific protein aggregates a further centrifugation step (30min, 21000g, 4°C) was carried out. The sepharose coupled antibodies were washed once with IP-buffer and incubated with cell extract for 2h at 4°C on a rotating wheel. After three wash cycles (two times with IP-buffer and once with wash-buffer) proteins were eluted from the drained sepharose matrix adding 20µl 4x SDS-sample buffer and analyzed by SDS-Page and immunoblotting.

2.2.29 *In vitro* protein-protein binding studies

For the *in vitro* protein binding studies purified GST alone or GST-FGF7 or FGFR2IIIb-D1-D3-IgG were applied. GST-FGF7 and GST, immobilized to a sepharose matrix were sequentially incubated with PLAC1 from overexpressing HEK293T cells and with FGFR2IIIb-D1-D3-IgG. After extensive washing with CoIP buffer, interacting proteins were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies.

2.2.30 Coupled *in vitro*- transcription and -translation of proteins

In vitro coupled transcription/translation of proteins was carried out using the "TNT Quick Coupled Transcription/Translation Reticulocyte Lysate System" according to the manufacturer´s protocol (Promega). Radioactive labeled L-[35S] Methionine was used.
Standard reaction:

1µg Plasmid DNA
2µl [35S]-Methionine (10 µCi/µl)
25µl TNT Quick Coupled Reticulocyte Lysate

Reactions were incubated for 90 min at 30°C and subsequently freeze at -20°C. An aliquot of the translation product was analyzed by SDS-PAGE and autoradiography.

Expression and purification of proteins

2.2.31 Production of secreted proteins in HEK293Tcells

Mammalian cells are suitable hosts for expression of properly folded proteins that possess post-translational modifications that might influence biological activity (e.g. glycosylation, sulfation, and phosphorylation). For recombinant protein production, HEK293T cells were transiently tansfected with DNA using PEI as previously described (2.2.18 Transient transfection of eukaryotic cells). 24h post-transfection cells were washed carefully with PBS and incubated with Pro293-T serum-free medium. After 96h the supernatant containing the protein of interest was collected and consequently processed for protein purification. To check for protein expression, samples of media were taken and analyzed by SDS-PAGE.

2.2.32 Affinity chromatography purification FC-fusion proteins

Specially, transmembrane proteins (e.g. receptors) are very difficult to purify, because of their hydrophobic membrane domains. To simplify this approach, fusion-proteins were generated. Therefore a certain domain (e.g. the extracellular domain of a receptor) was fused to the Fc region of an immunoglobulin. The Fc region comprises the CH2 and CH3 domains of the IgG heavy chain as well as a signal peptide that mediated the secretion of the protein. Cell culture supernatants of transfected HEK293T cells were collected 96h after transfection centrifuged and filtered through a 0.2µm filter. Fc-fusion proteins have been purified by Protein-A affinity chromatography with the Äkta purifier FPLC/HPLC- system as described by the manufacture. Prior to loading the sample, the column was equilibrated with 10 column volumes of binding

buffer. After loading, a wash step with binding buffer consisting of 10 column volumes was performed till no material appears in the effluent. FC-fusion proteins were eluted by acidic elution conditions from the Protein-A column. To archive the activity of acid labile fusion proteins 500µl of 1M Tris-HCl, pH 9.0, was added to the collection tubes, so that the final pH of the sample will be approximately neutral.
The purified fractions were buffer exchanged using HiTrap Desalting columns according to the manufacturer's protocol (GE Healthcare, Sweden).

2.2.33 Affinity chromatography purification of non-tagged proteins

The purification of the non-tagged Fibroblast growth factor FGF7 was performed using a HiTrap Heparin HP (5ml) column by affinity chromatography with the Äkta purifier FPLC/HPLC-system. Cell culture supernatant of FGF7 transfected HEK293T cells was centrifuged and filtered through a 0.2µm filter. Column was equilibrated with five column volumes of equilibration buffer. After sample loading, elution of bound proteins was achieved with a linear salt gradient (0.2 M – 2.0M NaCl). The collected fractions were analyzed by SDS-Page and Western blot with specific antibodies.

2.2.34 Production of recombinant proteins in *E. coli* cells

For recombinant protein expression the *E. coli* stain BL21 (DE3) was transformed with an adequate bacterial expression vector. A single colony was used to inoculate 500ml - 2l super broth-, TB- or LB- medium. During exponential growth phase (OD600 ~ 0.8) protein expression was induced with 1mM IPTG at 16°C. After 4-6h cells were harvest, washed once with PBS and stored at -20°C or consequently processed for protein purification. To check for protein expression samples were taken before and after induction and analyzed by SDS-PAGE.

2.2.35 Affinity chromatography purification of soluble GST-fusion proteins

Glutathione-*S*-transferase (GST) fusion proteins were purified from bacterial lysates by native affinity chromatography on glutathione-Sepharose. For protein purification cell pellets were resuspended in ice-cold GST-purification buffer (25µl buffer per ml culture) and lyzed by sonication (five times for 30sec each, with 1min rests between sonication). Intact cells, large cellular debris, and inclusion-body protein were pelleted by centrifugation (20min at 30000 rpm, 4°C). The supernatant containing the soluble fusion protein was incubated with 2ml of 50% slurry of equilibrated glutathione-Sepharose to each 100ml lysate for 2h at 4°C on an

orbital shaker. Finally the resin with the immobilized protein was washed four times with GST-purification buffer and stored at -80°C or consequently processed for protein-protein interaction studies. Elution of the GST-fusion protein from the glutathione sepharose was archived by gently resuspending the resin in glutathione buffer.

2.2.36 Size exclusion chromatography

Size exclusion chromatography (SEC) separates proteins on the basis of differences in molecular size. For this work size exclusion chromatography was employed to characterize the molecular dimensions of proteins, to determine the molecular weight of monomeric and multimeric states. Size exclusion chromatography was performed with prepacked columns (HiLoad 16/60 Superdex 200 prep grade, GE Healthcare). After calibration with reference samples (High and Low Molecular Weigh Standards for size exclusion chromatography) of known molecular size, chromatography with a soluble protein extract was performed. Prior to loading the proteins, the column was equilibrated with 2 column volumes of SEC-buffer. Protein extracts were loaded to the column so that proteins were separated based on their molecular size as they move through the porous matrix (smaller molecules have greater access and migrates slowly; larger molecules have less access and migrates faster). Fractions of 1.5ml were collected and analyzed by SDS-PAGE and Western blotting.

III RESULTS

3.1 Protein expression of the placenta specific protein PLAC1

3.1.1 Analysis of PLAC1 protein expression in different tissues and cell lines

The placenta specific protein, PLAC1, is an X-linked gene whose expression is restricted to the placenta as revealed by northern analysis (69) and *in situ* hybridization (72). No detectable expression of PLAC1 was observed in other normal adult or fetal tissues using these methods. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) confirm these observations and also demonstrated an expression but at much lower levels in testis (73). Recently, PLAC1 expression has been observed in a variety of human primary cancer types and cancer cell lines (68) indicating that PLAC1 might be a highly attractive target for immunotherapeutic therapies. Concerning these approaches and to support the obtained result from RT-PCR, protein expression of human PLAC1 was analyzed in a high variety of normal human tissue (Figure 9) and different human cancer cell lines, descending from different organs (Figure 10). To analyze protein expression of hPLAC1 in man a set of 30 normal tissue sections were re-suspended in 4x SDS-lysis buffer, lyzed by sonication and analyzed for hPLAC1 expression by SDS-Page and Western-blotting. As shown in Figure 9 hPLAC1 is only expressed in the placenta but is not detectable in any other normal tissue (upper panel, lane 15).

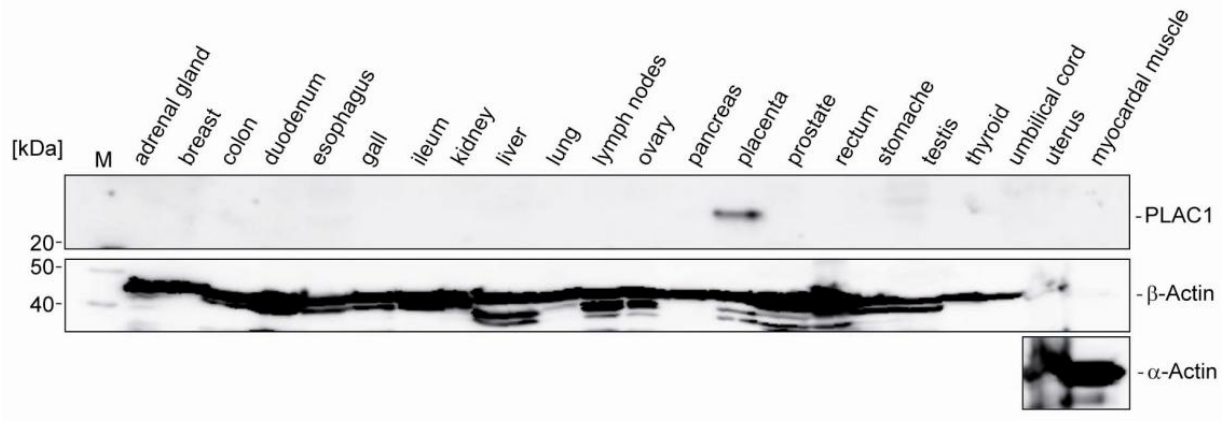

Figure 9. Expression of hPLAC1 in human tissues.
Total protein extracts were generated from 22 human tissues as indicated and normalized to β-Actin (middle panel). Actin from uterus and myocardial muscle were additionally analyzed by a α-Actin specific antibody as these tissues do not express high levels of β-Actin (lower panel). Expression of PLAC1 was analyzed by immunoblotting with specific antibodies (upper panel). PLAC1 is only expressed in placental tissue (lane 15).

β-Actin was used as loading control to normalize the extracts (middle panel). In the case of myocardial muscle and uterus no β-Actin signal was achieved, since in these tissues α-Actin and not β-Actin is expressed (lower panel). The results presented above clearly confirm the described expression pattern of hPLAC1 mRNA and show that hPLAC1 protein expression is restricted to placenta in man. Further, protein expression of hPLAC1 was analyzed in systematical screen using different cancer cell lines descending from different organs. To analyze hPLAC1 expression whole protein extracts from cell lines were generated as described in material & methods 2.2.21. Protein concentration was measured by protein absorbance at 280nm and same protein amounts were loaded on a SDS-Page and analyzed by immunoblotting with specific antibodies. β-Actin was used as a loading control to ensure equal loading (Figure 10, lower panels).

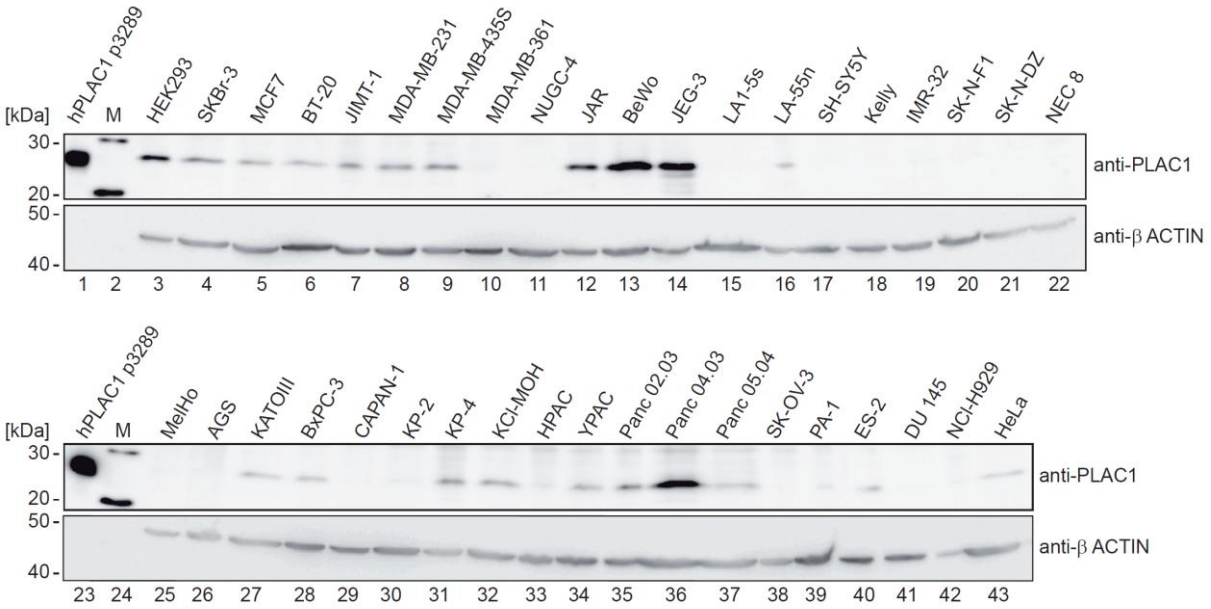

Figure 10. Comparison of PLAC1 expression level of human immortalized cell lines.
Total protein extracts were generated from 39 different cell lines as indicated and normalized to β-Actin (lower panel). Expression of hPLAC1 was analyzed by immunoblotting with specific antibodies (upper panel). HEK293, stably expressing human PLAC1 was used as control and for normalization (lane 1).

PLAC1 protein expression was observed in wide range of immortalized cell lines (Figure 10, upper panels). The highest expression level was detected in cell lines descending from a trophoblastic lineage: JAR, JEG-3 and BeWo (lane 12-14). But also the human pancreatic adenocarcinoma epithelial cell line, Panc04.03, showed comparable expression rates (lane 36). PLAC1 expression was also observed in human embryonic kidney (lane 3) and several human mammary gland carcinoma cell lines (lane 4-9) as well as in pancreatic cell lines

(lanes 31-35) but to a lower extent. Concluding from this systematical screen, PLAC1 expression was increased in cell lines of trophoblastic, breast and pancreatic lineage. These results were consistent with the data obtained after RT-PCR (68). For further cellular and biochemical analysis the trophoblast cell line BeWo was chosen, as expression analysis revealed highest expression in this cell line.

3.2 Localization studies of the placenta specific protein PLAC1

3.2.1 PLAC1 contains a functional signal peptide

Localization of a protein is one of the most important aspects to understand the function of a protein. Especially the localization of the human placenta specific protein 1 (hPLAC1) may play a crucial role considering its function in the placenta or in tumor as well as its potential importance for cancer immunotherapy. The localization of hPLAC1 is one of the most controversial discussed findings in the literature, describing hPLAC1 as transmembrane protein (68) or as a cytosolic protein with close proximity to the apical plasma membrane (74, 75). To clarify this topic, bioinformatical predictions for the existence of a signal peptide were performed using public available prediction algorithms (http://www.cbs.dtu.dk/services/SignalP/). SignalP consists of two different predictors based on neural network (NN) (Figure 10B) and hidden Markov model (HMM) (Figure 10C) algorithms (96-98). Both algorithms predict that hPLAC1 contains a signal peptide (aa 1-23) which targets the propeptide to the endoplasmic reticulum (ER) and that hPLAC1 is transported out of the cell by the secretory pathway.

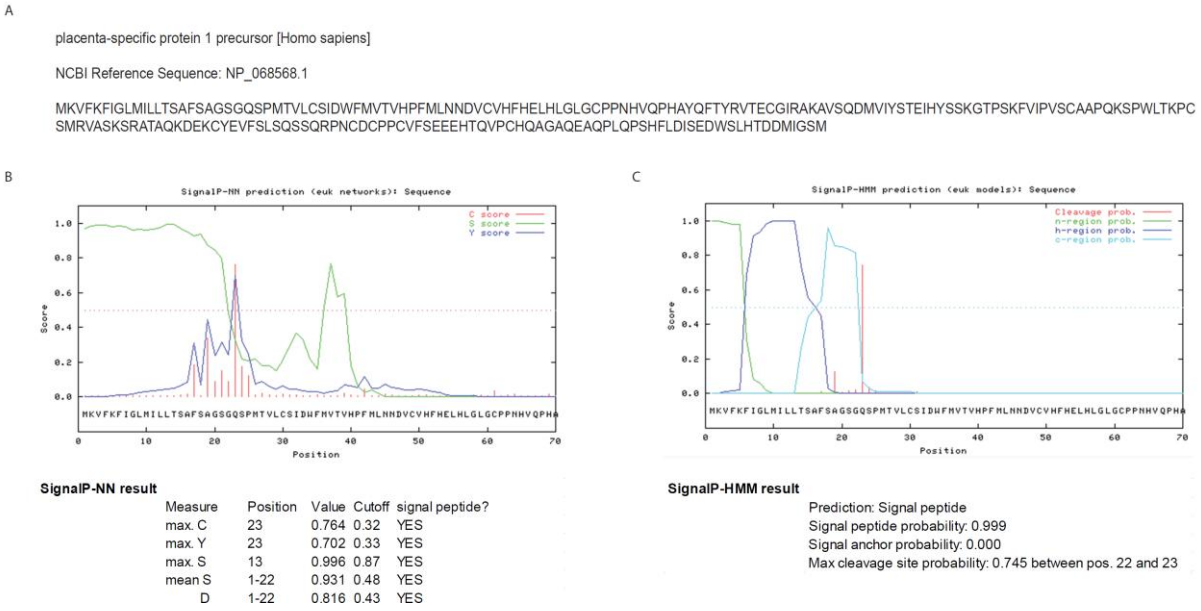

Figure 11. hPLAC1 contains a predicted signal peptide.
Predictions for a signal peptide applying the hPLAC1 amino acid sequence [A] have been made with the public available web server http://www.cbs.dtu.dk/services/SignalP/. SignalP consists of two different predictors based on neural network (NN) [B] and hidden Markov model (HMM) [C] algorithms (96-98).

To analyze, if hPLAC1 contains a functional signal peptide (SP) as deduce from the bioinformatical analysis, a ΔSP variant of hPLAC1 was generated. This variant was lacking the predicted signal sequence (aa 1-22) and thus it codes for a smaller protein beginning at aa 23 till aa 212. Furthermore, the predicted signal peptide of hPLAC1 (aa 1-22) was fused to GFP and to the hPLAC1 carboxy terminus (aa 116-212). A schema of the used constructs is depicted in Figure 12A. To analyze, if the signal peptide is cleaved off during protein processing, all constructs were transfected in HEK293T and proteins were analyzed 48h post-transfection by SDS-PAGE and Western blotting. In parallel, *in vitro* coupled translation of all proteins was carried out using the TNT quick coupled transcription/translation reticulocyte lysate system with radioactive labeled L-[^{35}S] methionine and analyzed by SDS-PAGE and autoradiography. In contrast to transfection were proteins undergo the normal cellular processing, the *in vitro* translated proteins are not processed further after translation. *In vitro* translated proteins reflect the original size of the translated protein. To analyze, if the signal peptide is cleaved off during protein processing, protein sizes were compared between proteins, undergoing an *in vivo* cellular processing with those which were *in vitro* translated. As shown in Figure 12B, hPLAC1 with or without a signal peptide (left panel, lane 1 and 2) have the same size after *in vivo* processing, although the wild type protein codes for 22aa more than the truncated variant without a signal peptide. In contrast, when proteins were *in vitro* translated a clear shift is detectable between hPLAC1 with or without a signal peptide (right panel, lane 7 and 8). This shift corresponds to the lacking 22aa in the case of the truncated hPLAC1 variant. The same effect was also observed in the case of GFP and the carboxy terminal truncation: GFP wt compared to GFP fused to the hPLAC1 signal peptide (left panel, lane 5 and 6; right panel, lane 11 and 12) as well as the carboxy terminal truncation of hPLAC1 (aa 116-212) with and without the signal peptide (left panel, lane 3 and 4; right panel, lane 9 and 10), migrated at the same size after *in vivo* expression but exhibit a shift after *in vitro* translation, respectively. This finding demonstrate, that hPLAC1 contains a functional signal peptide witch probably conducts the protein to the ER and is cleaved during protein processing.

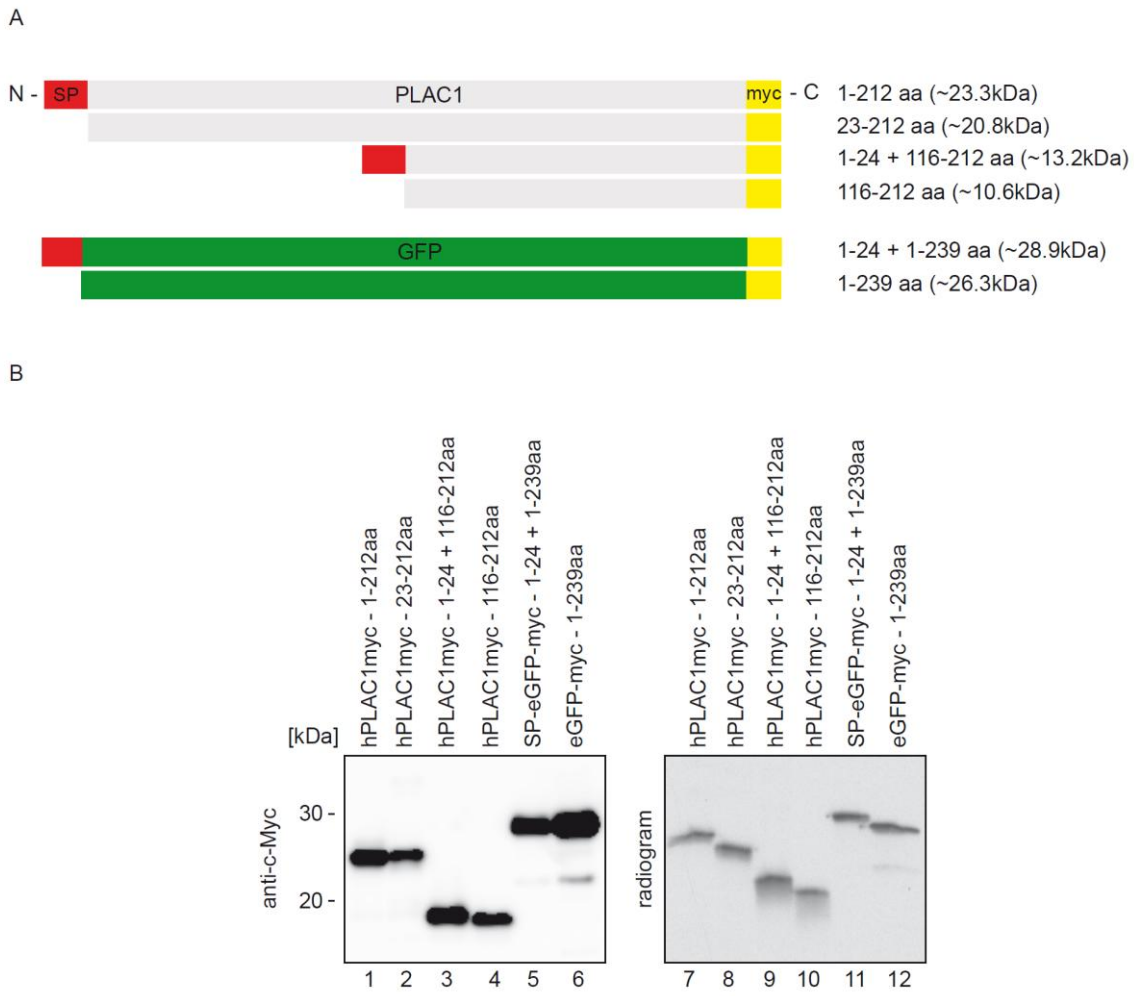

Figure 12. hPLAC1 contains a functional signal peptide.
A. Schematic illustration of different truncations used for the analysis of the hPLAC1 signal peptide function. The signal peptide is colored red, hPLAC1 in gray, eGFP in green and the myc-tag in yellow. B. hPLAC1 with (lane 1) or without (lane 2) signal peptide as well as C-terminal hPLAC1 truncations (aa 116-212aa) with (lane 3, 9) or without SP (lane 4, 10) and GFP wt (lane 5, 11) and mutant (lane 6, 12) were transiently transfected in HEK293T cells and migration of all forms were analyzed by SDS-PAGE and Western Blotting using tag specific myc antibodies (left panel). In parallel, all constructs were *in vitro* translated (right panel) using 35S-methionine. Migration differences between wt and truncated proteins were compared after *in vivo* or *in vitro* processing.

3.2.2 Fractionation of cell compartments of BeWo cells

To further support that hPLAC1 is a secreted protein and to analyze if hPLAC1 is associated with to the cell surface, the subcellular localization of endogenous hPLAC1 was analyzed in BeWo cells. For the selective isolation of the different cellular compartments (cytosol, membranes, nucleus and cytoskeleton) the "cell compartment kit" from Qiagen was used. This kit is designed for the subcellular fractionation of eukaryotic cells by sequential addition of different extraction buffers to a cell pellet.

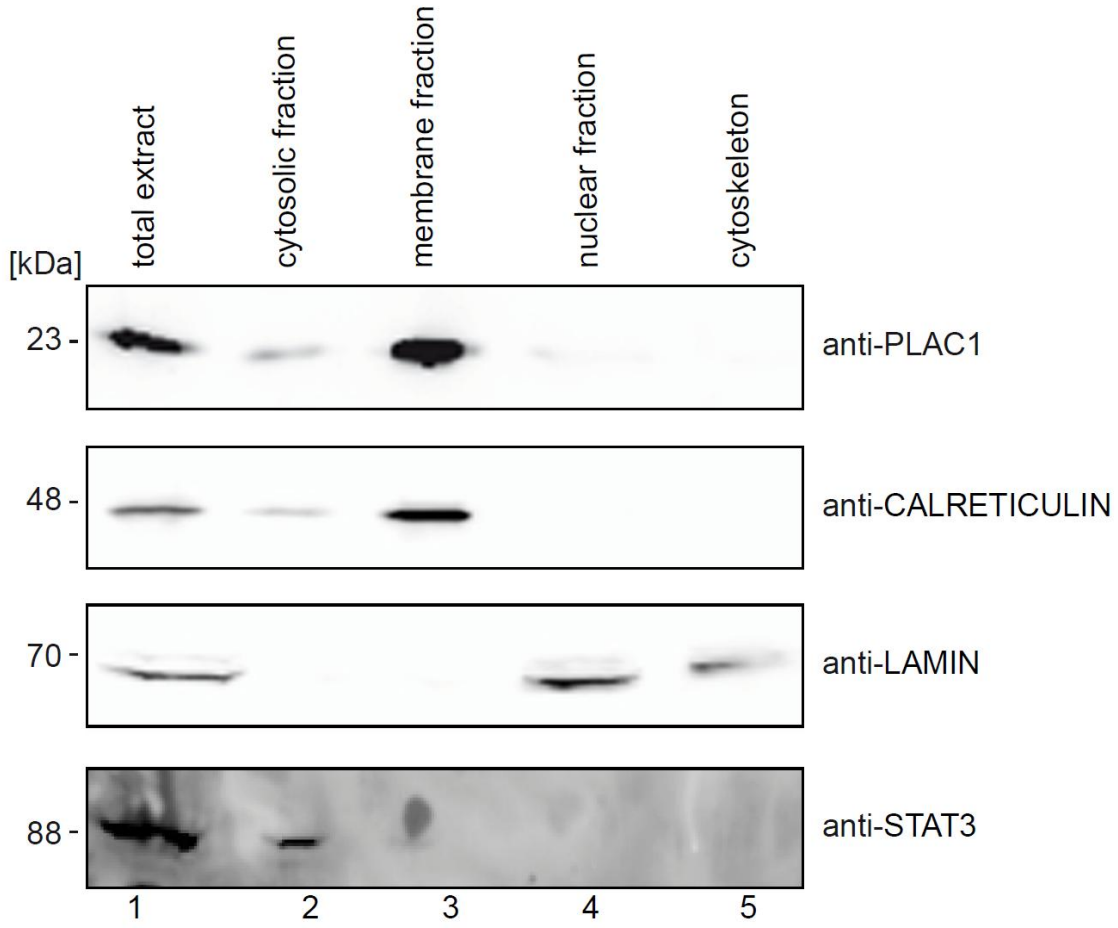

Figure 13. hPLAC1 is clearly enriched in the membrane fraction of BeWo cells.
The cell compartment kit (Qproteom Kit, Qiagen) was used for the subcellular fractionation of BeWo cells by sequential addition of different extraction buffers. Proteins in the different cellular compartments (cytosol, membranes, nucleus and cytoskeleton) can be selectively isolated and analyzed by SDS-PAGE and Western blotting. To control and validate the proper fractionation, different marker proteins of the cellular compartments were analyzed: cytosol-Stat3; membrane-calreticulin and nucleus-Lamin. A clear enrichment of hPLAC1 is detectable in the membrane fraction (lane3).

BeWo cells were carefully washed with cold PBS and incubated with the first buffer. The first buffer selectively disrupts the plasma membrane without solubilising it, for thus only cytosolic proteins result after centrifugation in the supernatant. Plasma membranes and compartmentalized organelles, such as nuclei, mitochondria and the endoplasmic reticulum (ER) remain intact and are pelleted by the first step. This pellet was further incubated in the second buffer, which solubilized the plasma membrane as well as all organelle membranes except the nuclear membrane. After solubilization the sample was centrifuged. This supernatant contains membrane proteins and proteins from the lumen of organelles (e.g., the ER and mitochondria). The pellet consists of nuclei, which are solubilized using the third buffer. After a final centrifugation, the fourth buffer solubilises all residual structures, mainly

cytoskeletal proteins of the remaining pellet. All obtained fractionations were analyzed by SDS-PAGE and Western blotting with PLAC1 specific antibodies. To validate the quality of the fractionation process, proteins specific for each compartment were analyzed by Western blotting. As shown in Figure 13, proteins specific of the different compartments were only detected in the accordant fraction: Calreticulin in the membrane fraction, Lamin in the nuclear fraction and Stat3 in the cytosolic fraction. For PLAC1 a clear enrichment was detectable in the membrane fraction. This finding support, that PLAC1 is associated to the membrane.

3.2.3 Secreted hPLAC1 is not detectable in the supernatant of transfected cells

Due to the secretion signal and the lack of TM-domain, it is likely that hPLAC1 is a soluble, secreted protein, detectable in the supernatant of cultured hPLAC1 expressing cells. To assess whether hPLAC1 is secreted, cells were transiently transfected and the supernatant was analyzed in a time course. As reference a well described secreted protein (hFGF7) and an intracellular protein (eGFP) were also analyzed. There for HEK293T cells were transfected either with hPLAC1, hFGF7, eGFP or even co-transfected and cultured in a time course from 0h-192h. At the indicated time points a sample of transfected cells as well as from the culture supernatant was analyzed by SDS-PAGE and Western blotting with specific antibodies (Figure 14). Moreover, total- as well as soluble or insoluble- proteins were analyzed in this time course analysis. First, the expression of the FGF7 protein, which should be secreted to the extracellular lumen, was analyzed as a positive control. FGF7 has been described to contain a signal peptide (aa 1-31) that mediates its secretion via the ER and Golgi pathway. During ER and Golgi processing hFGF7 pass through multiple modifications like O- as well as N-glycosylation till it is finally secreted to the extracellular lumen (99). As shown in Figure 14 (panel 1-4; row B and C), hFGF7 is clearly expressed in transfected cells. In addition hFGF7 is detectable in the supernatant of single or co-transfected cells (panel 2; rows B and C). The hFGF7 protein detected in the supernatant migrates slower in comparison to hFGF7 of total cell extracts, indicating the different glycosylations hFGF7 receive during processing. The obtained results clearly demonstrated that hFGF7 is a secreted protein. Further, the expression of cytosolic protein eGFP, which should not be secreted to the extracellular lumen, was analyzed as a negative control. eGFP is clearly expressed by transfected cells (panel 1-4; row F). Surprisingly, eGFP was also detected at low levels in the supernatant of transfected cells (panel 2; row F), although it should not be detected in the supernatant since eGFP is a cytosolic protein and is not secreted to the extracellular lumen. Probably this portion of eGFP detected in the supernatant might derive to apoptotic cells or to broken cells during harvest.

The results obtained for hPLAC1 expression and secretion are shown in Figure 14 panel 1-2; row A, D and E. hPLAC1 is expressed after transfection and is still detectable 192h post-transfection in total protein lysates. However, PLAC1 was not detectable in the supernatant.

As mentioned above, cellular expression levels were differentiated between total protein lysates and soluble- or insoluble- protein lysates. Analysis of total protein lysates revealed high expression levels of all analyzed proteins (hPLAC1, hFGF7 and eGFP) (panel 1; row A-F). After generating soluble- / insoluble protein lysates (panel 3 and 4; row A-F) the main fraction of the reference proteins (hFGF7 and eGFP) got solubilized (compare panel 3 and 4; row B, C and F). In contrast hPLAC1 was mostly detected in the insoluble fraction (panel 3 and 4; row A, D and E). These results indicate that hPLAC1 is a highly insoluble protein.

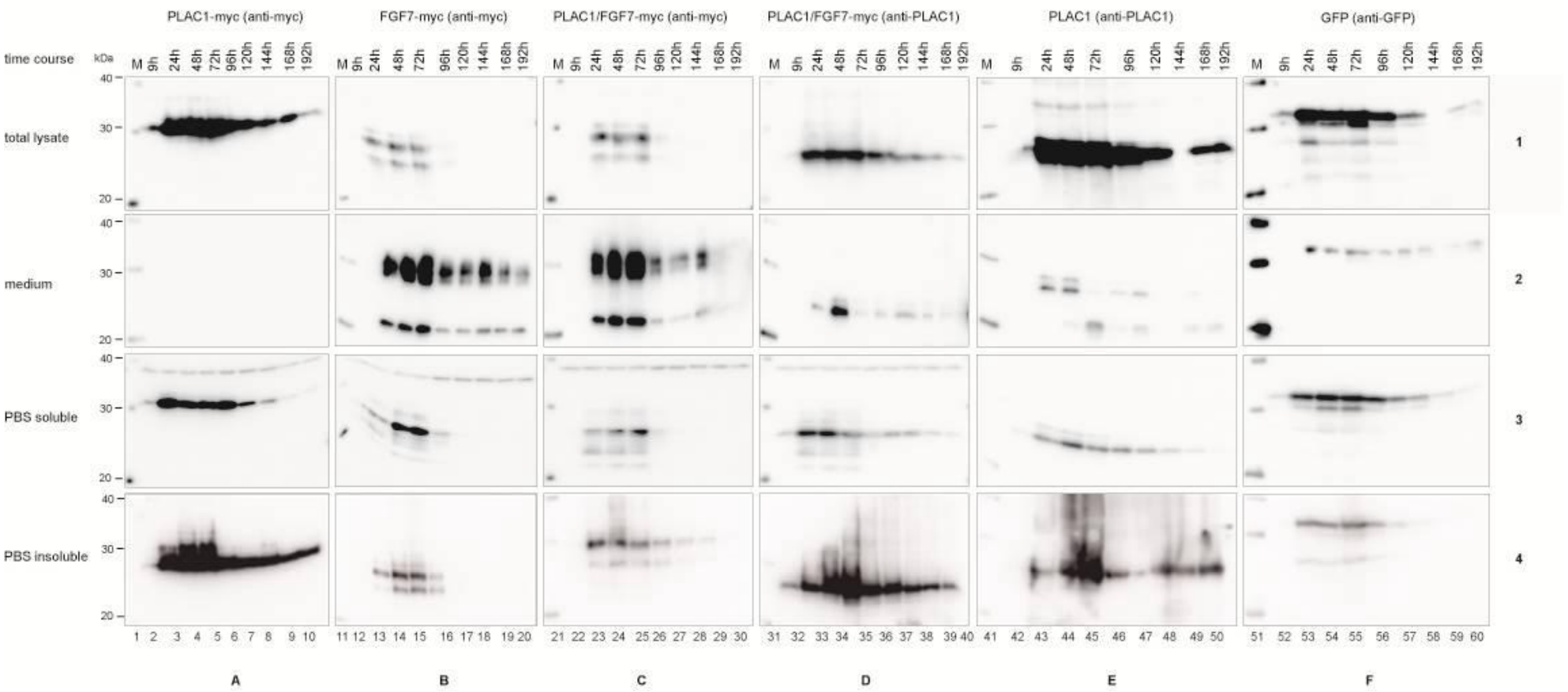

Figure 14. hPLAC1 is not secreted in the supernatant when it is overexpressed.
The expression and secretion was analyzed in a HEK293T overexpressing hPLAC1. A time course was performed. Protein expression of whole protein extracts (1), soluble and insoluble lysate-fractions (3 - 4) and secretion to the media (2) was analyzed by Western blotting with respective antibodies. hFGF7 was used as example of a well described secreted protein (B). To analyze the expression of a cytosolic protein eGFP was used. Further, eGFP was used to analysis the contribution of apoptotic or broken cells that mimic a false signal in the supernatant of eGFP overexpressing cells.

3.3 Supramolecular organization of the placenta specific protein PLAC1

3.3.1 PLAC1 forms supramolecular structures

The placenta specific protein, PLAC1, was described to share significant homology (30% identity and 45% similarity, P=0.0018) to *zona pellucida* 3 proteins (ZP3) (69). ZP3 proteins are specific sperm-binding glycoproteins, also referred to as sperm receptors, in the *zona pellucida*. The *zona pellucida* (ZP) is a specialized extracellular matrix that surrounds the oocyte and the early embryo. The ZP is composed of four glycoproteins (ZP1-4) with various functions during oogenesis, fertilization and preimplantation development (77, 78). The presence of that structural element has been found in hundreds of extracellular proteins of diverse functions. ZP proteins consist of two subdomains: the N-terminal (ZP-N) and the C-terminal (ZP-C) domain. However, the amino-terminal part (ZP-N) is described as a module responsible for the formation of filaments or subramolecular structures and might be responsible for protein polymerization due to disulfide bonds formation of cysteine residues (76, 81). Four of six cysteine residues within the ZP-N domain of human and murine PLAC1 are conserved within different species and correspond to cysteine residues 1–4 of the ZP domain (79). Leading to the assumption that hPLAC1 may contain a ZP-N motive as well as that it might form multimeric structures, size exclusion chromatography was performed. Size exclusion chromatography separates proteins on the basis of differences in molecular size due to the stokes radius of a protein in aqueous solution, so that molecular dimensions of proteins or the molecular weight of monomeric and multimeric states of a protein can be determined. The size exclusion chromatography was performed with a protein extract from BeWo cells, endogenously expressing PLAC1 (Figure 15A). In a second separation an extract from HEK p3289, stably overexpressing PLAC1 was used (Figure 15B). The HEK293 p3289 cell line constitutively overexpress the genomically integrated construct p3289, which codes for a full length, untagged human PLAC1 protein (aa 1-212). The stable expressing cell line HEK293 p3289 was generated by subcloning and replacing the standard media with media containing G418 selection marker. The stable expressing cell line HEK293 p3289 was kindly provided by Ganymed Pharmaceuticals AG. Protein extracts were generated as previously described, were loaded on the column and separated based on their molecular size.

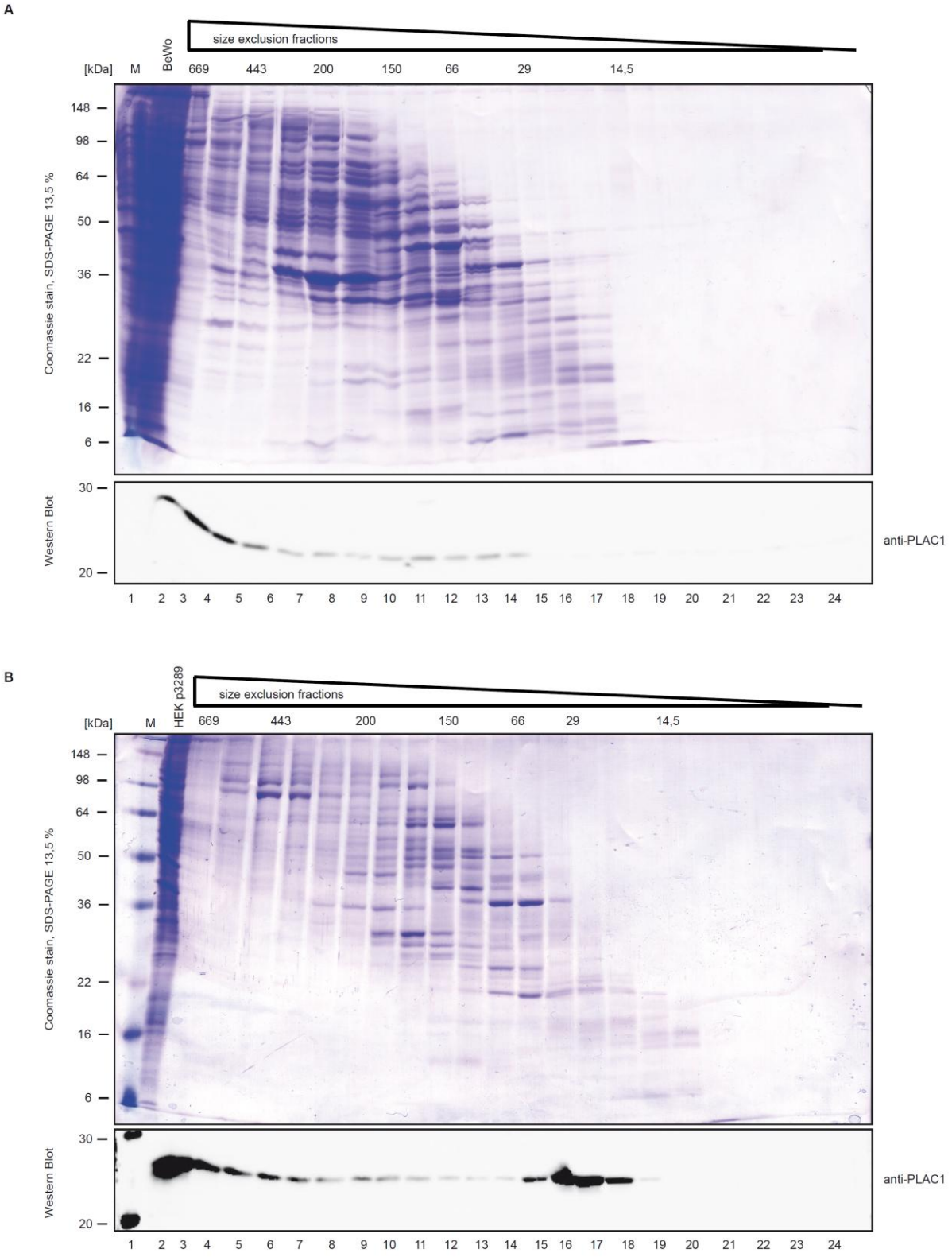

Figure 15. hPLAC1 in size exclusion chromatography.
Protein extract from BeWo cells (A) and HEK p3289 cells (B), were separated by analytical size exclusion chromatography. Size exclusion chromatography was performed with prepacked columns with an Äkta Purifier System. Obtained fractions were analyzed by SDS-PAGE and Western blotting with specific antibodies. For calibration of the column, a molecular weight standard from GE Healthcare was used. Lane 2 indicates total cell extract, prior to separation.

Subsequently, fractions of 1.5ml were collected and analyzed by SDS-PAGE and Western blotting. Coomassie staining of separated size exclusion fractions of BeWo or HEK p3289 extract show a clear separation of proteins based on their size (Figure 15 A+B, upper panels). Western blotting with specific PLAC1 antibodies results in a diffuse signal pattern over all fractions (Figure 15 A+B, lower panels). This result clearly demonstrated that hPLAC1 is able to form low and high molecular structures: hPLAC1 molecules in fraction 15-18 correspond to monomeric hPLAC1 molecules, given that calibration of the column shows that proteins between 50-25kDa got fractionated in this area. Fractions 3-14 correspond to high molecular weight proteins (669-100kDa). PLAC1 molecules detected in these fractions may correspond to multimeric structures (lane 3-14). Furthermore, these multimeric structures could also be observed after separation on polyacrylamide gels and electroblotting with a tank transfer unit (Figure 16). Whole protein extracts of HEK293T cells overexpressing human PLAC1 (aa 1-212) or macaque PLAC1 (aa 1-212) were generated using 4xSDS-lysisbuffer and analyzed by Western blotting with three different monoclonal antibodies.

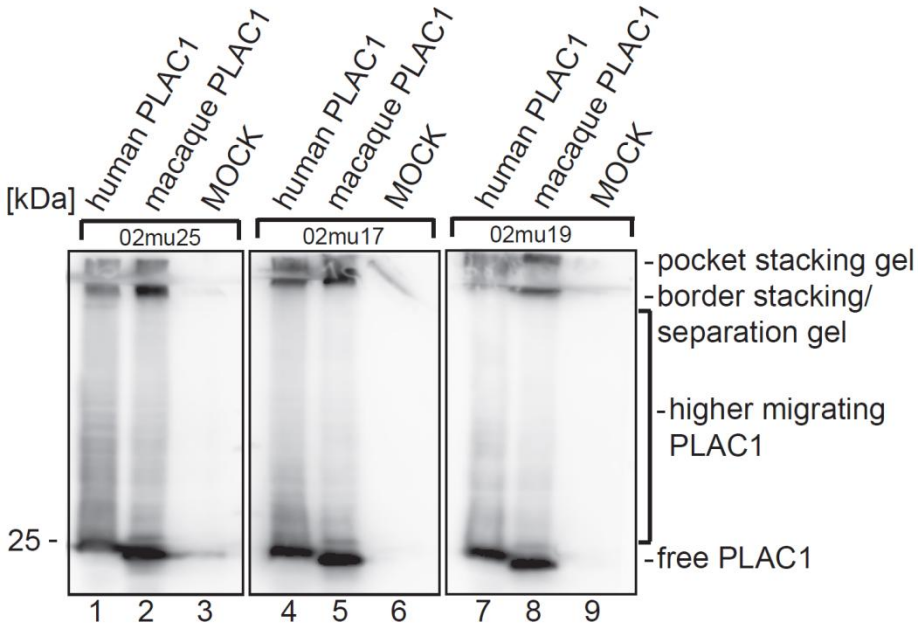

Figure 16. PLAC1 can form higher migrating, covalently linked complexes in different species.
Total cell extracts from HEK293T cells, overexpressing human (lane 1, 4 and 7) or macaque (lane 2, 5 and 8) PLAC1 were separated by SDS-PAGE and analyzed by Western blotting. Mock transfected HEK293T cells were used as control (lane 3, 6 and 9). For detection of PLAC1, different specific PLAC1 antibodies recognizing different epitopes within the PLAC1 molecule were used.

Each of that monoclonal antibodies recognized different epitopes within the PLAC1 protein. Predominantly monomeric human and macaque PLAC1 was detected at the expected size (~25kDa) but also a smear of PLAC1 was observed over the whole separating lane. Further, accumulation of PLAC1 at the border of stacking and separation gel and in the pocket of the stacking gel was observed. This indicates that multi- and subramolecular structures are present in the protein extract. Theses subramolecular structures are probably formed by covalent disulfide bonds or non-covalent bonds. However, these subramolecular structures seem to be partially resistant to the reducing agent β-mercaptoethanol, to bowling and to the denaturing agent SDS (see more discussion). Together with the results obtained after size exclusion chromatography, it seems obvious that PLAC1 is able to form high molecular structures, ranking from dimeric to multimeric molecules, representing a mixture of covalently and non-covalently linked homo- or heteromeric interactions.

3.3.2 PLAC1 forms homodimers *in vivo*.

The predicted putative ZP-domain within PLAC1 and the results shown above prompted to analyze if hPLAC1 can homodimerize. Dimerization of PLAC1 might lead to a behavior that results in multimeric structures as described for other ZP-like proteins (76, 79). To this end, co-immunoprecipitation experiment were performed using HA- or myc- tagged PLAC1. HEK293T cells were single or co-transfected with the respective plasmid DNA and co-immunoprecipitations were performed 48h post-transfection. Proteins were immunoprecipitated from extracts with either HA- or myc-antibodies (Figure 17A-B), immobilized to NHS-sepharose. Precipitations were analyzed by Western blotting with respective antibodies. PLAC1-HA was specifically precipitated by the coupled HA-antibodies (lane 6 and 7) but not by the control antibodies (lane 9 and 10). To verify that PLAC1 forms homodimers co-precipitation of PLAC1-myc was analyzed by Western blotting (Figure 6 A, lower panel): PLAC1-myc specifically interacts with PLAC1-HA (lane 7) and thereby forms homodimers. The same result was achieved in a vice versa experimental setup (Figure 6B): Immunoprecipitation with an anti-c-Myc coupled antibody results in a co-precipitation of PLAC1-HA (lower panel, lane 7). This result clearly demonstrates for the first time, that PLAC1 forms homodimers *in vivo*.

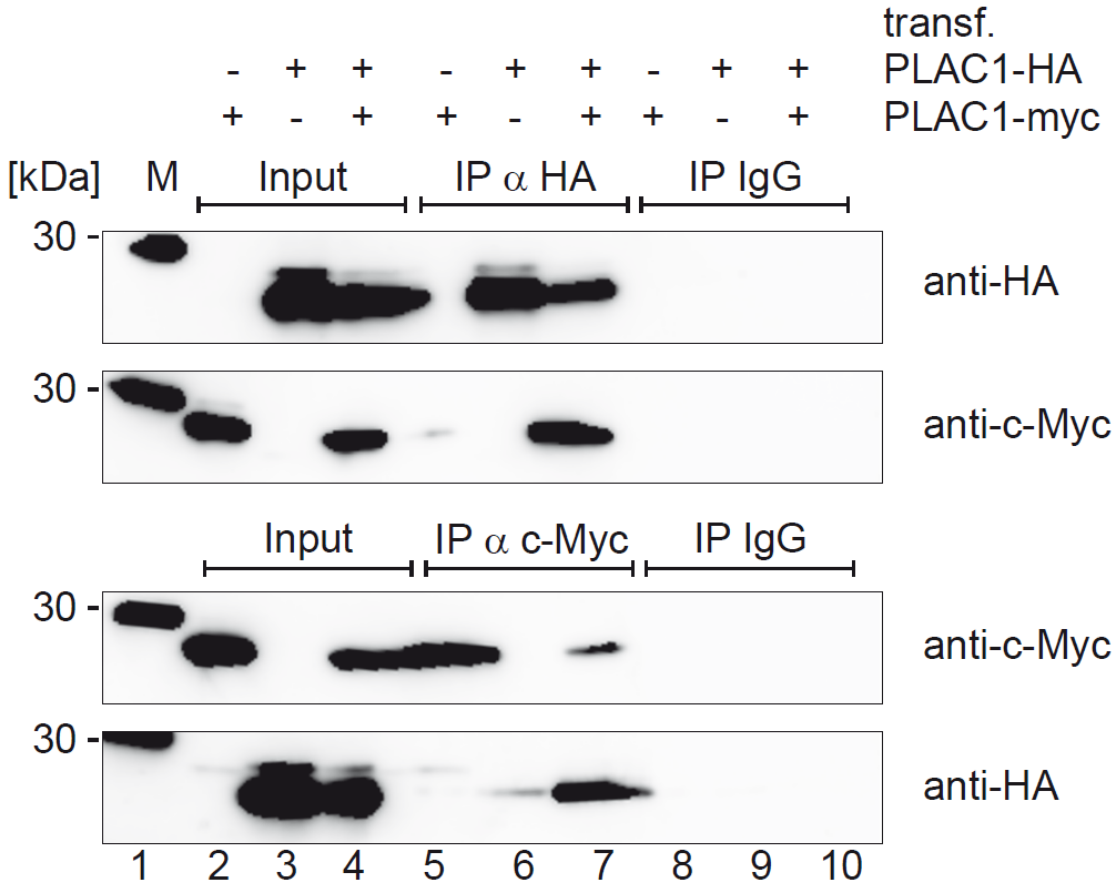

Figure 17. hPLAC1 forms homodimers *in vivo*.
HA- and myc- tagged PLAC1 were single or co-transfected in HEK293T cells. PLAC1 was immunoprecipitated from extracts with either HA- (upper panel, lanes 5-7) or myc-antibodies (lower panel, lanes 5 -7), immobilized to NHS-sepharose. As control IgG1 was used (lanes 8-10). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies.

3.3.3 PLAC1 forms homodimers via its N-terminal part

The dimerization of hPLAC1 described above as well as published data (76) provide evidence that the PLAC1 homology region of the ZP-N domain is necessary and sufficient for protein polymerization. In the case of human PLAC1 and murine Plac1 this ZP-N motif localizes between amino acids 89 and 106 (76). To support the published *in vitro* studies, we performed binding studies *in vivo*. Therefore, wild type (aa 1-212) and three truncated PLAC1 variants (aa 23-212; aa 1-122; aa 116-212) were subcloned into mammalian expression vectors. A schematic illustration of the truncation used in this experimental setup are depicted in Figure 18A. *In vivo* binding studies were performed with wild type HA-tagged PLAC1 and myc-tagged variants. HEK293T cells were co-transfected with the respective plasmid DNA and 48h post-transfection, co-immunoprecipitations were performed (Figure 18B). PLAC1-HA

was specifically precipitated by the coupled HA-antibodies (lower panel, lane 1- 4) but not by the control antibodies (lower panel, lane 5-8). To verify that PLAC1 forms homodimers via the amino-terminus, co-precipitation of full length or truncated PLAC1-myc mutants were analyzed by Western blotting (upper panel, lane 1-4). Only the truncated protein, coding for the C-terminal part of hPLAC1 (aa 1-24+116-212) did not interact with PLAC1-HA (aa 1-212) (lane 4). Full length (aa 1-212) and the N-terminal truncated variants (aa 23-212 and aa 1-122) still were capable to form homodimers with PLAC1-HA (aa 1-212) (lane 1, 2 and 3). Considering that wild type as well as these N-terminal truncations of hPLAC1 carries the homology region of the ZP-N domain, these binding studies approve that PLAC1 forms homodimers via the PLAC1-homology region of the ZP-N domain *in vivo*.

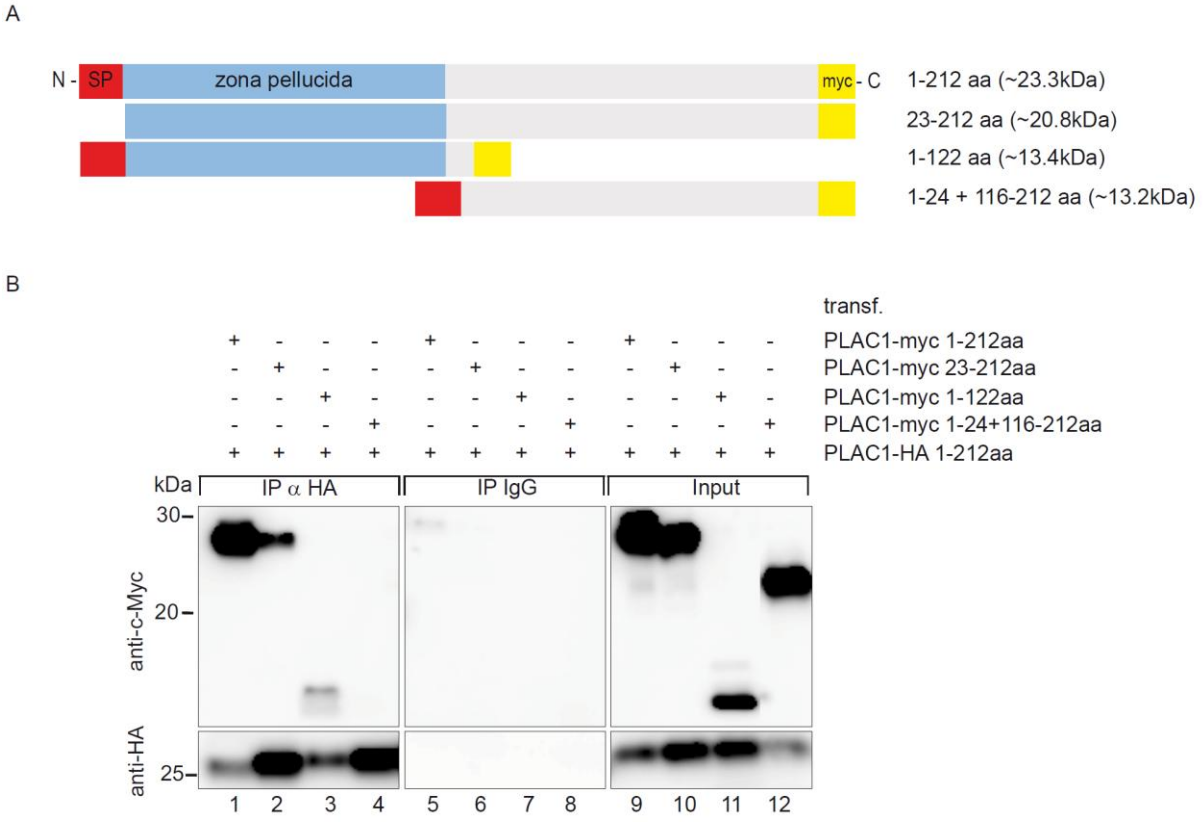

Figure 18. hPLAC1 can form homodimers via its N-terminal ZP-N-domain.
A. Schematic illustration of different truncations used for the binding studies. The signal peptide is colored in red, hPLAC1 in gray and the myc-tag in yellow. The predicted ZP-N domain is colored in blue B. HA- and myc- tagged hPLAC1 truncations were co-transfected in HEK293T cells. hPLAC1-HA was immunoprecipitated from extracts with HA- (lanes 1-4), immobilized to NHS-sepharose. As control IgG was used (lanes 5-8). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies. Lanes 9-12 represent the input used for precipitation assays.

3.4 Interaction partners of the placenta specific protein PLAC1

3.4.1 PLAC1 specifically interacts with the fibroblast growth factor FGF7

The role that a protein adopts is often affected by its interacting network. Interactions are crucial interplays between proteins of the whole cellular and extracellular environment. Molecular pathways which are affected by these interactions as well as the interacting proteins themselves are often analyzed to understand the function of a protein. In the case of hPLAC1 the fibroblast growth factor 7 (FGF7), also referred to as keratinocyte growth factor (KGF), was identified to have a regulative function concerning the expression of hPLAC1. Fant et al described, that the level of hPLAC1 mRNA increased upon treatment of cells with FGF7 (72). FGF7 is a secreted glycoprotein that belongs to the fibroblast growth factor family. The FGF family consists of 23 members, which mediate their cellular responses by binding to and activating different isoforms of the four receptor tyrosine kinases FGFR1-4. So far the FGF signaling pathway has been characterized to play a significant role in normal development and wound healing but also in tumor development and progression (100-102). Specially FGF7 has been described to exhibit mitogenic functions and has a significant expression in estradiol-receptor positive breast- and pancreatic- carcinoma cell lines (103-106). Considering that both proteins, PLAC1 and FGF7, are secreted to the extracellular matrix, are expresses in breast and pancreatic carcinomas and are involved in tumor development, a direct interaction between PLAC1 and FGF7 was hypothesized. Interaction studies were performed by co-immunoprecipitations of both proteins. Therefore untagged hPLAC1 and hFGF7 were single or co-transfected in HEK293T cells and 48h post-transfection co-immunoprecipitations were performed (Figure 19). hFGF7 (A) as well as hPLAC1 (B) was specifically precipitated by the corresponding antibodies (lane 4-6) but not by control antibodies (lane 7-9). Binding of hPLAC1 was observed by precipitating hFGF7 (Figure 19A, lower panel, lane 6) as well as vice versa (Figure 19A, upper panel, lane 6). This co-immunoprecipitation clearly demonstrated that both proteins can interact *in vivo*.

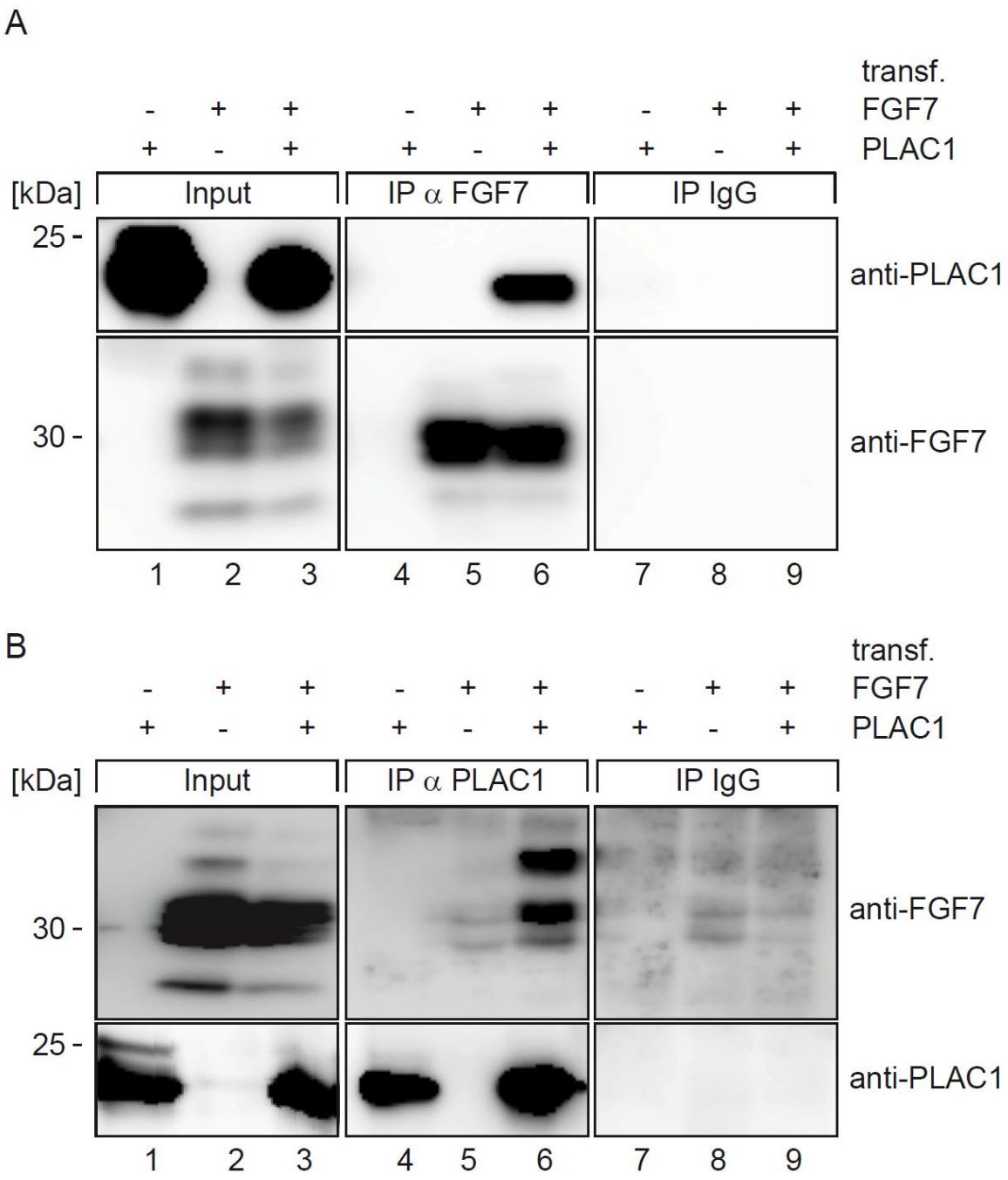

Figure 19. hPLAC1 interacts with hFGF7 *in vivo*.
hFGF7 and hPLAC1 were single or co-transfected in HEK293T cells. hFGF7 or hPLAC1 was immunoprecipitated from extracts with either FGF7- (upper panel, lanes 4-6) or PLAC1- specific antibodies (lower panel, lanes 4-6), immobilized to NHS-sepharose. As control corresponding IgG were used (lanes 7-9). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies.

As mentioned before the FGF family consist of 23 members, which range in a molecular mass from 17 to 34 kDa and share 13-71% amino acid identity and are highly conserved in gene structure and amino-acid sequence between vertebrate species (Figure 20; (99)).

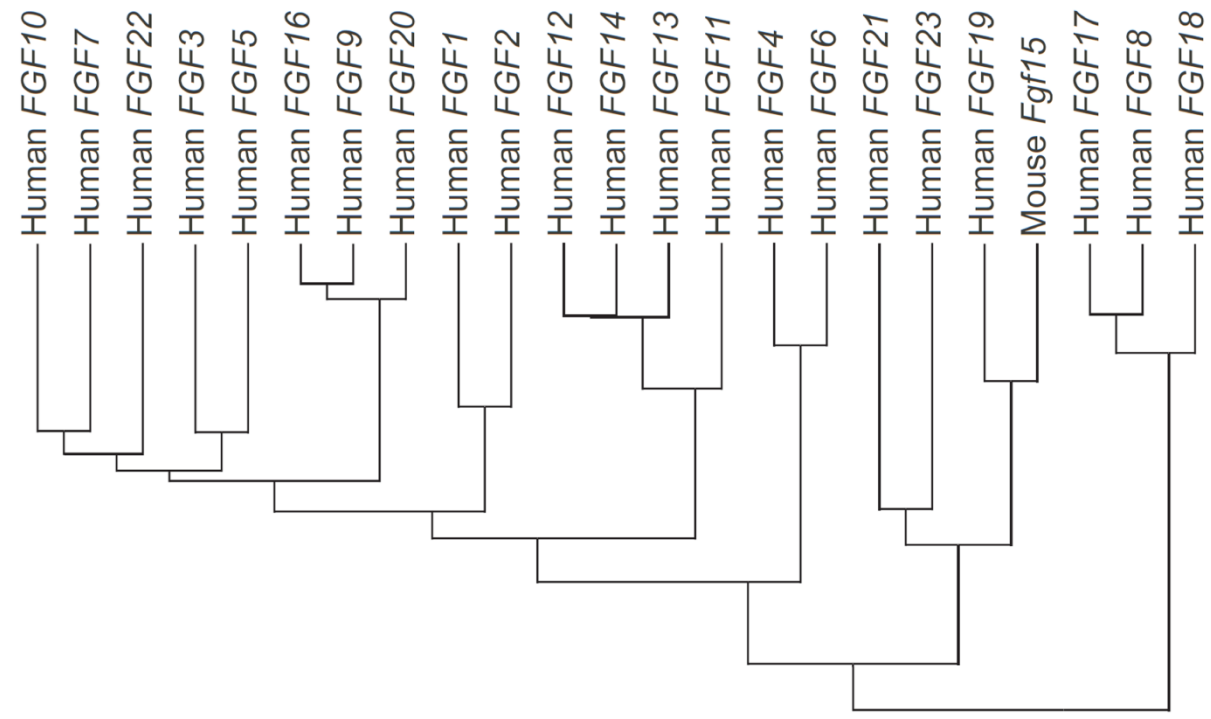

Figure 20. Evolutionary relationships within the FGF family.
Apparent evolutionary relationships of the 22 known human and murine FGFs. Sequences were lined using Genetyx sequence analysis software and trees were constructed from the alignments using the neighbor-joining method (99).

To analyze if hPLAC1 is a specific interactor of hFGF7 or if hPLAC1 is a general FGF binding partner, several members of the FGF family were used for co-immunoprecipitation studies. To this end FGF- 1, 2, 3, 7, 8 and 10, all tagged with a myc-tag at their C-terminus, were co-transfected with hPLAC1 in HEK293T cells. Binding studies were performed 48h post-transfection, using a PLAC1 specific antibody mix. Co-precipitating proteins were analyzed by Western blotting using an anti-c-Myc antibody (Figure 21). To this end, only hFGF7 directly interacts with hPLAC1 (lane 17). Concluding from these results, hPLAC1 seems to be specifically involved in the hFGF7 signaling network.

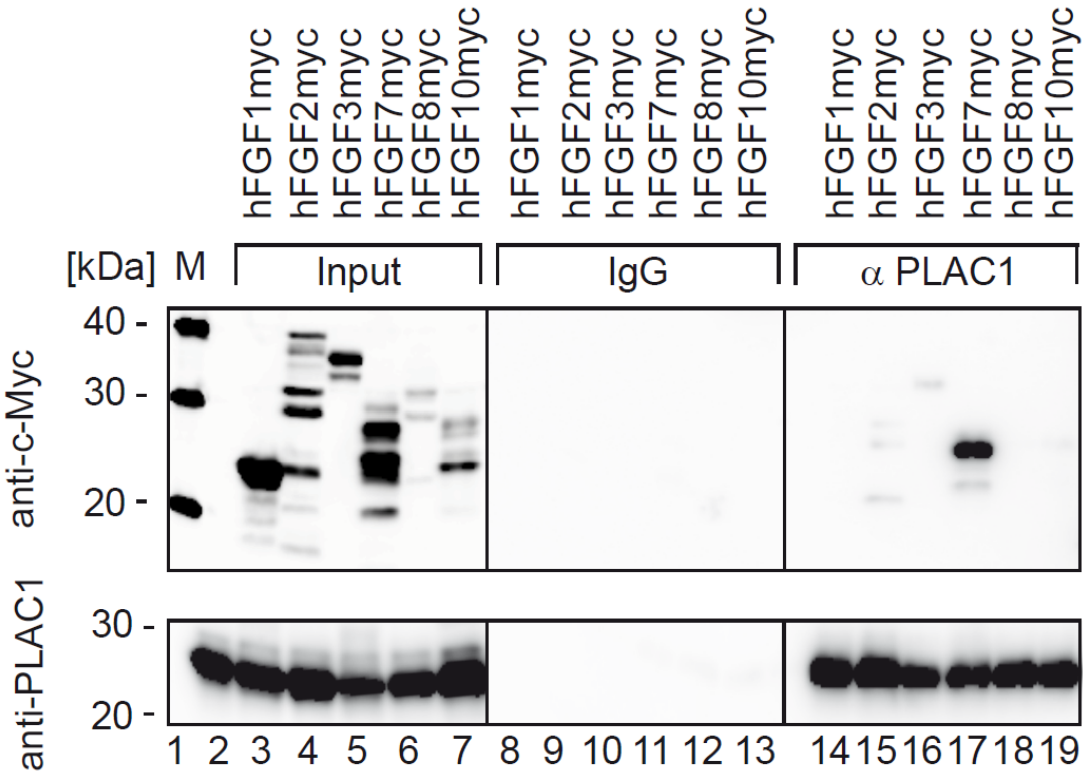

Figure 21. hPLAC1 specifically interacts with hFGF7.
hPLAC1 and five members of the FGF-protein family were co-transfected in HEK293T as indicated. hPLAC1 was precipitated with a monoclonal antibody mix, covalently linked to NHS-sepharose (lane 14-19). An unrelated mouse serum, covalently linked to NHS-sepharose was used as control (IP control, lane 8-13). Interacting proteins were eluted by boiling in SDS sample buffer, separated by SDS-PAGE and analyzed by immunoblotting using an anti-myc antibody. Lane 2-7 shows input of co-transfected cells.

3.4.1.1 The PLAC1-FGF7 interaction is conserved in different species

To address, if the observed interaction of PLAC1 with FGF7 is restricted to human or if the interaction is conserved in different species, the interaction between FGF7 and PLAC1 was analyzed with human, mouse and macaque PLAC1. Therefore PLAC1 cDNA of human, mouse and macaque were co-transfected with human FGF7 in HEK293T cells and co-immunoprecipitations were performed. The human and macaque PLAC1 open reading frame (ORF) encodes a putative protein of 212 amino acids, whereas the mouse Plac1 encodes a highly homologous but shorter product of 173 amino acids (Figure 22A). The ORF of FGF7 encodes a putative protein of 194 amino acids and is highly conserved in different species (Figure 22B).

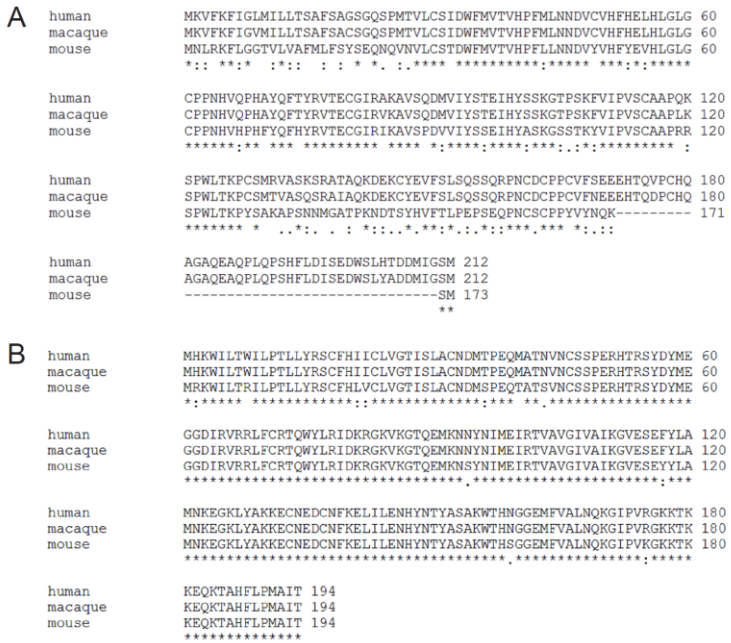

Figure 22. Multiple sequence alignment using the human, mouse and macaque PLAC1 [A] and FGF7 [B] sequence. Amino acid sequence of human, mouse and macaque PLAC1 [A] and FGF7 [B], were aligned with the public available web server http://www.ebi.ac.uk/Tools/msa/clustalw2/.

As shown in Figure 23 the interaction between PLAC1 and FGF7 is conserved in different species as observed by immunoprecipitations with an anti-FGF7 antibody. All three PLAC1 proteins co-precipitated with human FGF7 (upper panel, lane 1-3). Unspecific binding of mouse Plac1 could also be observed in control precipitations (upper panel, lane 5), but it is not significant when compared to the co-precipitation signals (upper panel, lane 2). In summary, the results presented above demonstrate that the PLAC1-FGF7 interaction is conserved in different species. Further as detected from the study above, a putative interacting domain of PLAC1 is not located within aa 174 - 212 as this C-terminal domain is lacking in mouse Plac1.

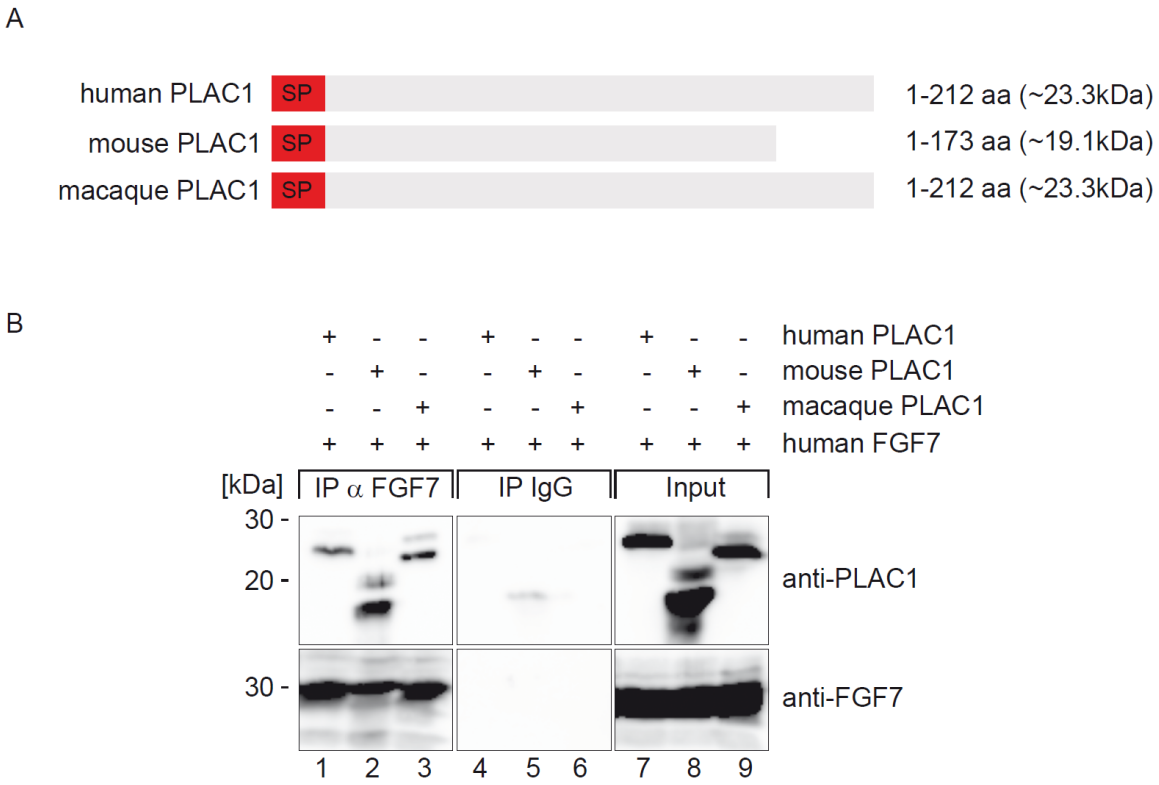

Figure 23. The interaction of PLAC1 and hFGF7 is conserved in different species.
To analyze, if the interaction of PLAC1 and hFGF7 is conserved, PLAC1 from three different species (mouse, macaque, human) were transiently co-transfected with human FGF7 in HEK293T cells. PLAC1 was co-immunoprecipitated from extracts by a FGF7 antibodies (lanes 1-3) immobilized to NHS-sepharose. As control IgG was used (lanes 4-6). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies. In lane 7-9 the input of cellular extracts is shown.

3.4.1.2 PLAC1 - FG7 interaction in placental cell lines

Regarding that PLAC1, FGF7 and the FGF7 specific receptor (FGFR2IIIb) are expressed in placental syncytiotrophoblast as demonstrated by immunohistochemistry (Figure 24, kind regards to Dr. Christoph Rohde) and that a placenta-specific KGF-axis was described to regulate trophoblast development (89), evidences were given, that the observed interaction between hPLAC1 and hFGF7 might also play an important role during placentation (87, 88, 107-111). The interaction between the placenta specific protein PLAC1 and the fibroblast growth factor FGF7 was observed when both proteins were overexpressed in HEK293T. To analyze if this interaction can be detected in endogenously expressing cells, BeWo cells were used for immunoprecipitations. The human placental choriocarcinoma cell line, BeWo, was chosen because of the endogenous expression of PLAC1 as well as FGF7 and the FGF7 specific receptor (FGFR2IIIb). Often only very low levels of KGF are detected in tumor cell lines, because of a loss of expression (105).

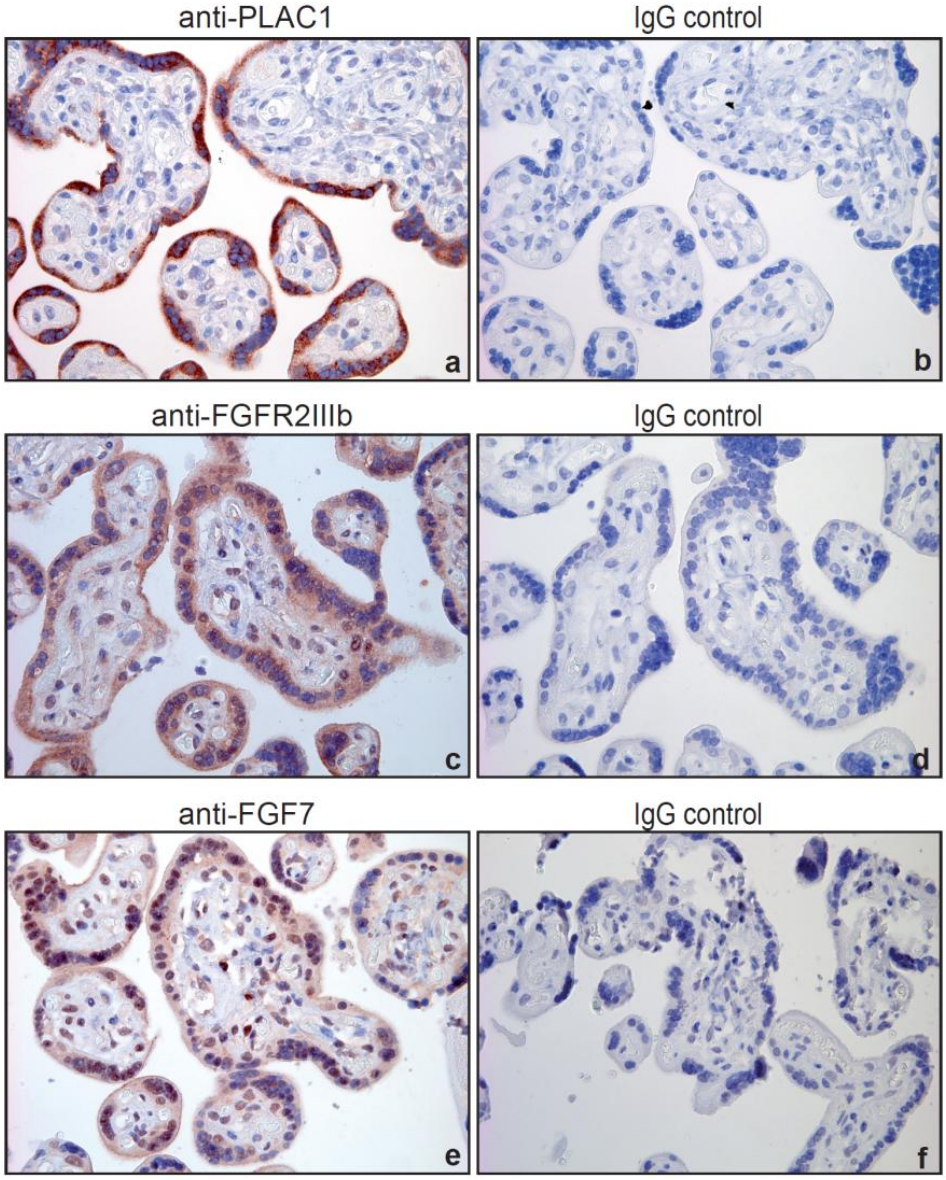

Figure 24. hPLAC1 co-localizes with hFGF7 and hFGFRIIIb in the syncytiotrophoblast of human placenta.
3µm thick sections of formalin-fixed and paraffin embedded human placenta were prepared. Sections were incubated with antibodies specific for PLAC1 (a), FGFR2IIIb (c) or FGF7 (e). Specific stainings are detected in the syncytiotrophoblast (red staining) with all three antibodies. As control the primary antibody was omitted and sections were incubated with the respective secondary antibody only (see b, d, f). Objective magnification for all panels: 40x.

To bypass this, BeWo cells were transiently transfected with an hFGF7 coding cDNA and binding studies were performed as described in material and methods 2.2.28. FGF7 was precipitated by a specific antibody. Binding of endogenous hPLAC1 was analyzed by Western blotting. In Figure 25, results of this semi-endogenous interaction study are shown. Co-precipitation of the interacting protein, hPLAC1, was detected (upper panel, lane 3). However, some portion of hPLAC1 also precipitated on control beads (upper panel, lane 4). Nevertheless, this signal was much weaker in comparison to the Co-IP (upper panel, lane 3

and 4). Concluding, the interaction between hPLAC1 and hFGF7 could be shown in overexpression (Figure 19) as well as in semi endogenous binding studies (Figure 25). These findings strengthen the point, that hPLAC1 might be involved in the previously described placenta-specific KGF-axis.

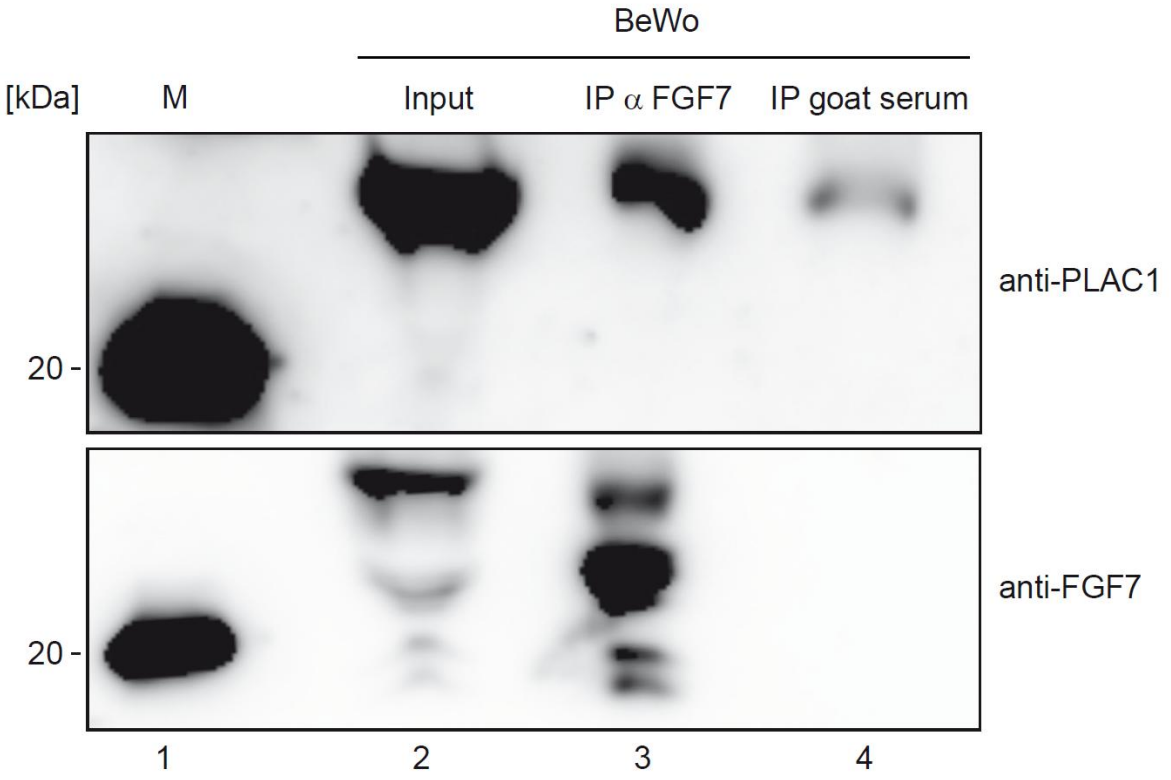

Figure 25. hPLAC1 interacts with hFGF7 *in vivo*.
The endogenous hPLAC1 expressing cell line BeWo was transfected with hFGF7. hFGF7 was immunoprecipitated from extracts with specific antibodies (lane 3), immobilized to NHS-sepharose. As control corresponding IgG were used (lane 4). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies. In lane 2 the input of cellular extracts is shown.

3.4.1.3 The placenta specific protein PLAC1 interacts with the fibroblast growth factor FGF7 via at least two independent binding sites.

To further analyze and characterize the binding of hPLAC1 and hFGF7, the specific binding domain for this interaction was determined. The mapping of the hFGF7 binding domain in hPLAC1 is of special interest considering immunotherapeutical approaches: Perturbation of the interaction by an antibody could inhibit the potential mitogenic effects of hFGF7. To analyze the binding motif, wild type (aa 1-212) and four truncated versions of hPLAC1 (aa 23-212; aa 1-122; aa 116-212 and aa 1-24+116-212) were subcloned into mammalian expression vectors: The first truncation of hPLAC1 consist of a protein without the predicted signal peptide, the second one encodes only the N-terminus, the C-terminus is expressed by

the third one and the fourth truncated protein consist of the C-terminus with an upstream hPLAC1 signal peptide. A schematic illustration of mutants used in this experimental setup is depicted in Figure 26A. HEK293T cells were co-transfected with the respective plasmid DNA and 48h post-transfection, *in vivo* binding studies were performed (Figure 26B).

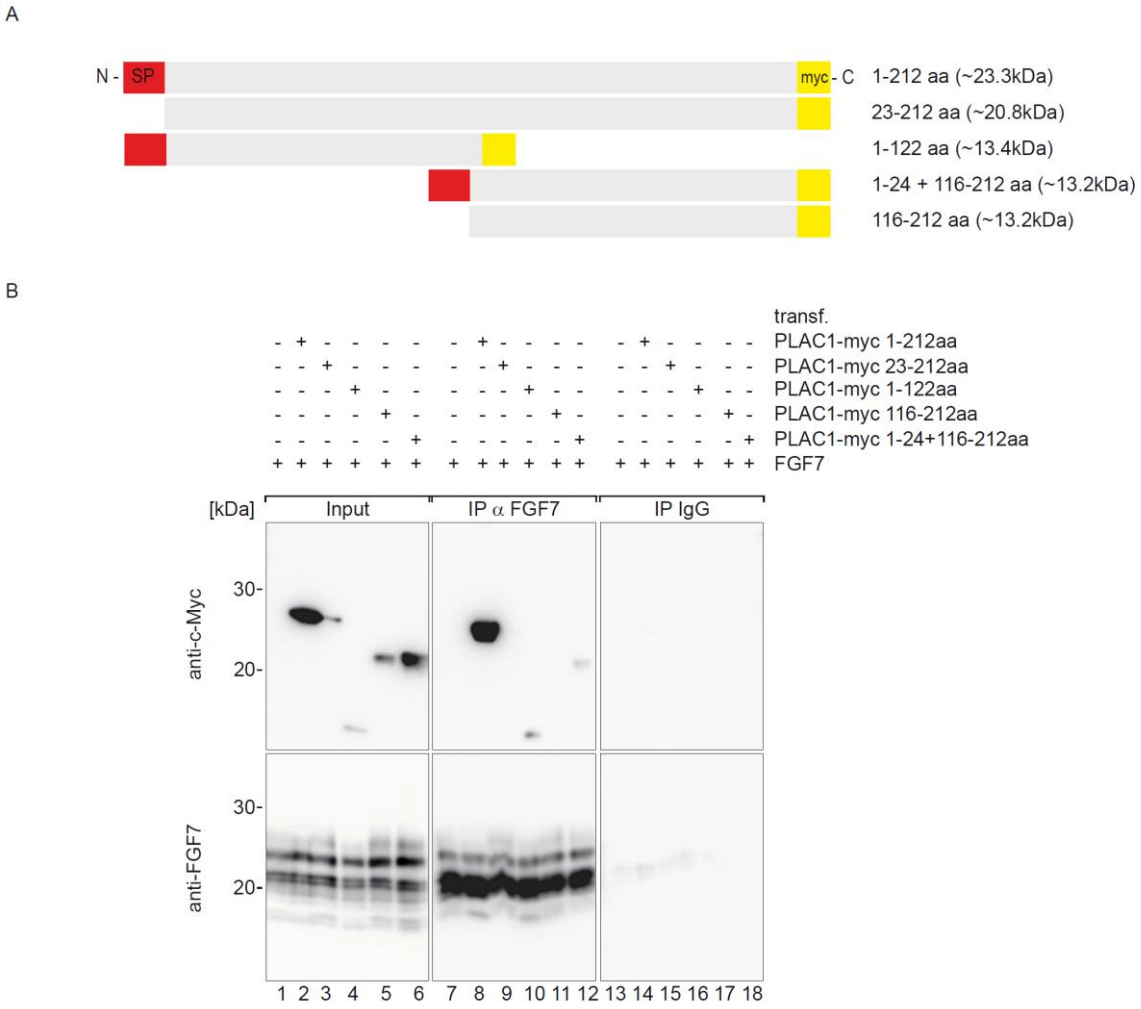

Figure 26. PLAC1 interacts via the N- and C- terminus with FGF7 *in vivo*.
A. Schematic illustration of different truncations used for the binding studies. The signal peptide is colored in red, hPLAC1 in gray and the myc-tag in yellow. B. hPLAC1 WT and truncated forms were co-transfected with hFGF7 in HEK293T cells. hFGF7 was immunoprecipitated from extracts with specific antibodies (lower panel, lanes 7-12), immobilized to NHS-sepharose. As control IgG was used (lanes 13-18). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies.

To determine the interacting domain between hPLAC1 and hFGF7, co-precipitation of full length or truncated hPLAC1-myc variants were analyzed by Western blotting (upper panel, lane 7-12). In contrast to the hPLAC1 protein lacking the predicted signal peptide (aa 23-212), full length hPLAC1 (aa 1-212) showed a strong interaction with hFGF7 (upper panel lane 8 and 9). The same results were obtained by the protein coding for the amino-terminus (aa 1-122). However, the protein encoding the hPLAC1 carboxy-terminus (aa 116-212) did

not interact with hFGF7 (upper panel, lane 11). Interestingly, when the predicted signal peptide of hPLAC1 (aa1-24) was fused to the C-terminus (aa 1-24+116-212), the interaction with hFGF7 could be recovered (upper panel, lane 12). These results lead to the assumption that the N- as well as the C-terminus of hPLAC1 is not sufficient for binding to hFGF7 on their own. Anyhow, the availability of a signal peptide was crucial for the interaction: truncated proteins without the signal peptide did not show an interaction with hFGF7. If the signal peptide of hPLAC1 promotes the interaction itself or if cellular processing is essential for the PLAC1-FGF7 interaction was analyzed.

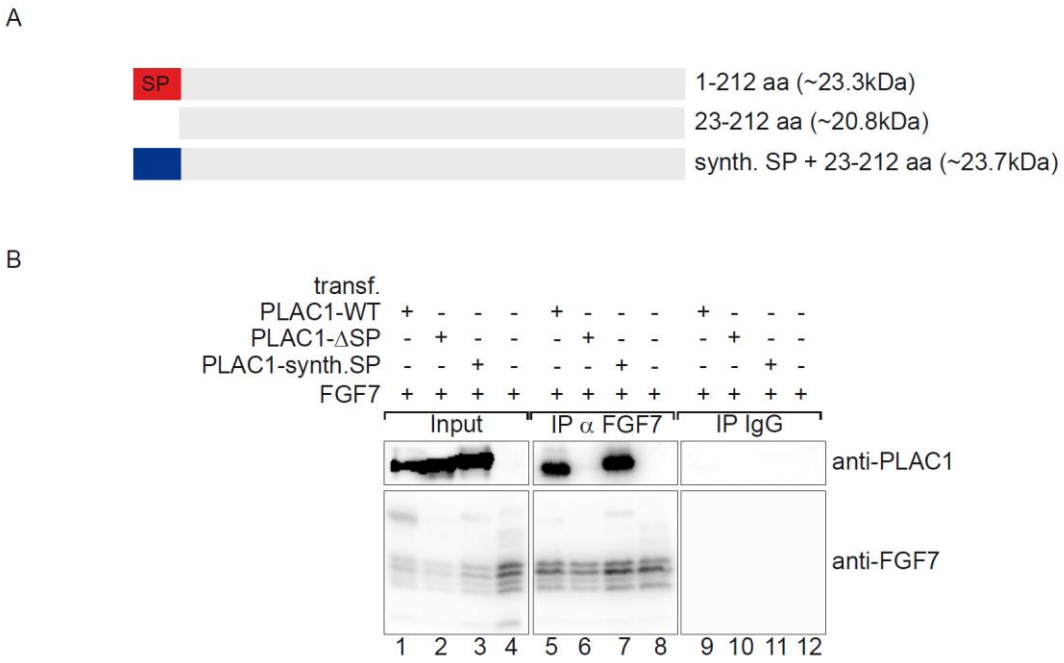

Figure 27. The export of hPLAC1 via ER and Golgi is a prerequisite for its interaction with hFGF7.
A. Schematic illustration of different truncations used for the binding studies. The PLAC1 signal peptide is colored in red, the autonomous kappa chain signal peptide in blue and hPLAC1 in gray. B. hPLAC1 WT (lane 1, 5, 9) without (ΔSP; lane 2, 6, 10) or with the kappa chain signal peptide (synth. SP; lane 3, 7, 11) were co-transfected with hFGF7 in HEK293T cells. hFGF7 was immunoprecipitated from extracts with specific antibodies (lanes 5-8), covalently linked to NHS-sepharose. As control, an unrelated goat serum was used (lanes 9-12). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies. Lane 1-4 show input of co-transfected cells.

To address this question the predicted signal peptide of hPLAC1 was substituted by the kappa chain signal peptide. In Figure 27A a schematic illustration of the generated hPLAC1 versions is depicted. Co-immunoprecipitations were performed as described before and hPLAC1 binding was analyzed by Western blotting (Figure 27B). PLAC1-WT and PLAC1 with the autonomous kappa chain signal peptide (hPLAC1 synth.SP) were able to interact with hFGF7 (upper panel, lane 5 and 7). In contrast, hPLAC1 lacking the signal peptide failed to interact

with hFGF7 (upper panel, lane 6). This binding study clearly demonstrates that the amino acids 1-24 of hPLAC1 are not involved in the PLAC1-FGF7 interaction by they own. This data presented above clearly demonstrate that the export of hPLAC1, mediated by its signal peptide, via ER and Golgi is a prerequisite for its interaction with hFGF7. Since it was not possible to determine the binding motif of hFGF7 by using the N- or C-terminal part of hPLAC1 (Figure 26), a set of further truncations, all containing a signal peptide, were generated. A schematic illustration of the generated truncations is depicted in Figure 28A. For binding studies, HEK293T cells were transiently co-transfected with hFGF7 and a truncated hPLAC1 cDNA. Co-immunoprecipitations were performed 48h post-transfection and finally analyzed by Western blotting (Figure 28B). Consecutively truncating the protein about 15 amino acids from the N- or the C-terminus did not affect the interaction with hFGF7. All truncated variants still show an interaction, confirming previous obtained results were amino- and carboxy-terminus are involved in hFGF7 binding.

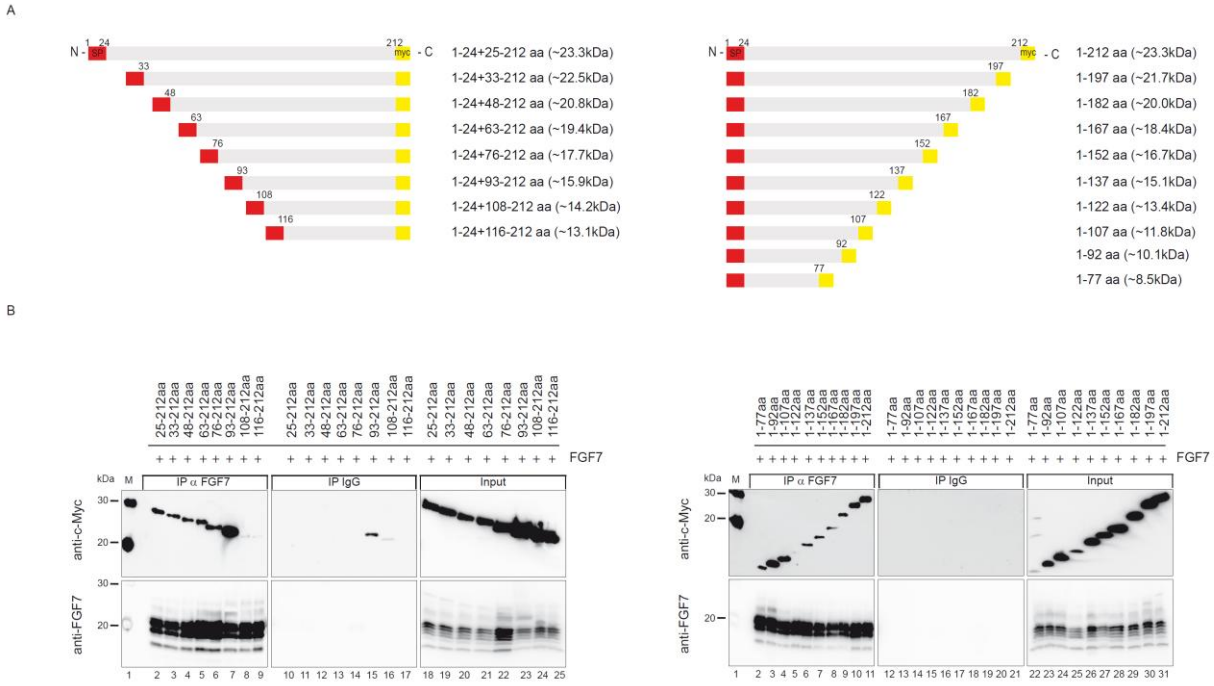

Figure 28. hPLAC1 interacts via the N- and C- terminus with hFGF7 *in vivo*.
A. Schematic illustration of different truncations used for the binding studies. The signal peptide is colored in red, hPLAC1 in gray and the myc-tag in yellow. B. hPLAC1 WT and truncated forms were co-transfected with hFGF7 in HEK293T cells. hFGF7 was immunoprecipitated from extracts with specific antibodies, immobilized to NHS-sepharose. As control IgG was used. Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies.

To further verify that the amino- as well as the carboxy-terminus of hPLAC1 are involved in hFGF7 binding intermediate truncations of hPLAC1, lacking the N- and C-terminal part of the protein, were generated: The so called variant mu1 coding for aa 92-182 and the variant mu2 coding for aa 108-182. In Figure 29A a schematic illustration of these constructs is depicted. Co-immunoprecipitations were performed with extracts of HEK293T co-transfected cells and binding of these both truncations of hPLAC1 to hFGF7 was analyzed by Western blotting. As shown in Figure 29B only full length hPLAC1 interacts with hFGF7 (upper panel, lane 8). In contrast, both intermediated mutants were clearly expressed but did not show an interaction with FGF7 (upper panel, lane 6 and 7; 10 and 11). With these and the previous presented results, it seems clear that human PLAC1 possesses at least two or more FGF7 binding sites, probably within its N- and C-terminus. Anyhow, each of these binding sites is able to interact independently with FGF7.

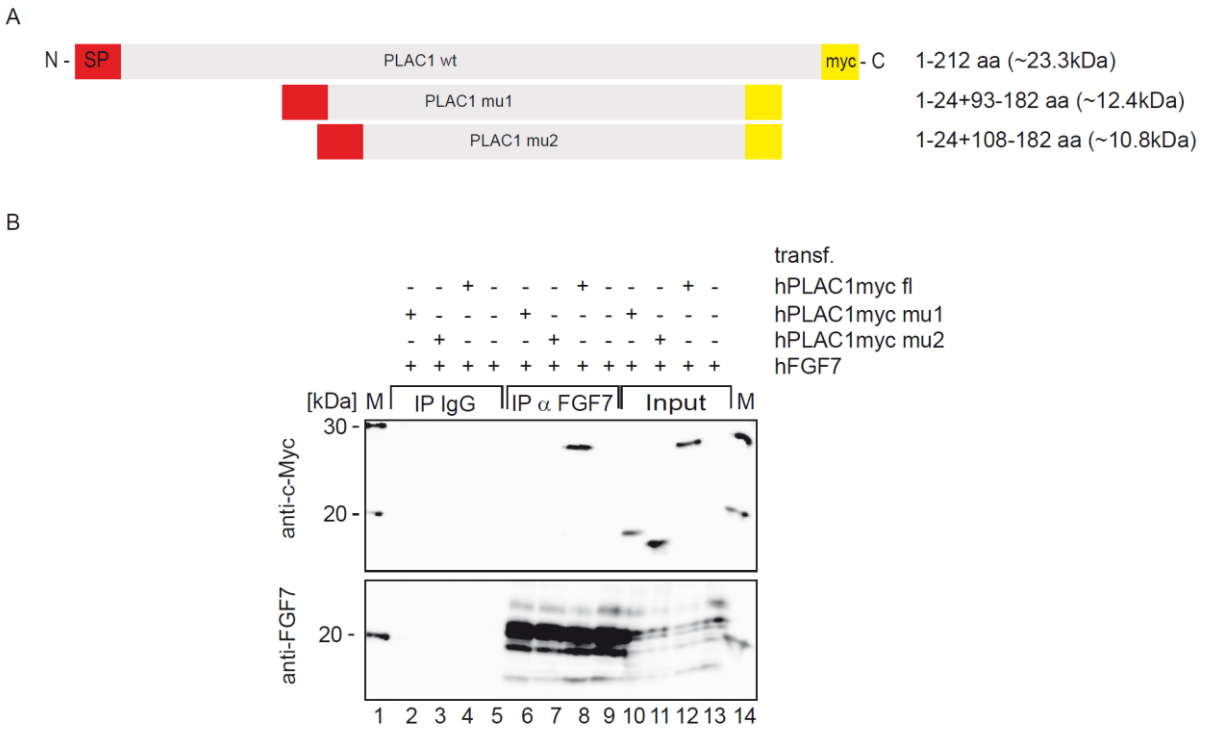

Figure 29. PLAC1 interacts with FGF7 via the N- and C- terminus *in vivo*.
A. Schematic illustration of different truncations used for the binding studies. The signal peptide is colored in red, hPLAC1 in gray and the myc-tag in yellow. B. hPLAC1 WT and truncated forms were co-transfected with hFGF7 in HEK293T cells. hFGF7 was immunoprecipitated from extracts with specific antibodies (lower panel, lanes 6-10), immobilized to NHS-sepharose. As control IgG was used (lanes 2-5). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies.

3.4.2 The placenta specific protein PLAC1 interacts with the glycosaminoglycan heparin.

The most abundant heteropolysaccharides of the body are the glycosaminoglycans (GAGs). These molecules are long unbranched polysaccharides, 36-470kDa in size, containing a repeating disaccharide unit. GAGs are located on the surface of cells or in the extracellular matrix (ECM). The specific GAGs of physiological significance are hyaluronic acid, dermatan sulfate, chondroitin sulfate, heparin, heparan sulfate, and keratan sulfate. Specially the polysaccharides heparin and heparan sulfate were described to play important roles in cancer and developmental biology, since they can bind to a wide range of proteins e.g. fibroblast growth factors and modulate their biological activity (112, 113). As presented above hPLAC1 also locates on the surface of cells and interacts with the fibroblast growth factor FGF7 (Figure 19), so a putative binding to the polysaccharide heparin was analyzed. Extracts of transfected HEK293T cells either with hPLAC1 or hFGF7 (as positive control) was used for pull-down experiments. The assay was performed with heparin cross linked to agarose and as negative control mock agarose matrix was used. Precipitations were analyzed by immunoblotting with specific antibodies (Figure 30A). hPLAC1 was specifically precipitated by heparin-agarose (lane 2) but not by the mock-agarose (lane 3). Furthermore, binding efficiency of hPLAC1 to heparin was comparable to hFGF7, which is a well characterized heparin-binding protein (lane 6). The interaction between the placenta specific protein PLAC1 and the polysaccharide heparin has been observed after overexpressing hPLAC1 in HEK293T. To analyze, if this interaction can be detected in endogenously hPLAC1 expressing cells, BeWo, Jeg-3, Jar and Panc04.03 cells were used. Pull down assays with heparin cross linked to agarose or mock agarose were performed with extracts derivated from these endogenously hPLAC1 expressing cell lines. Precipitations were resolved by SDS-PAGE and analyzed by immunoblotting. As shown in Figure 30B, the interaction between hPLAC1 and heparin was confirmed in all endogenous PLAC1 expressing cell lines. Taken together these experiments described above, clearly demonstrated that hPLAC1 is a new heparin-binding protein.

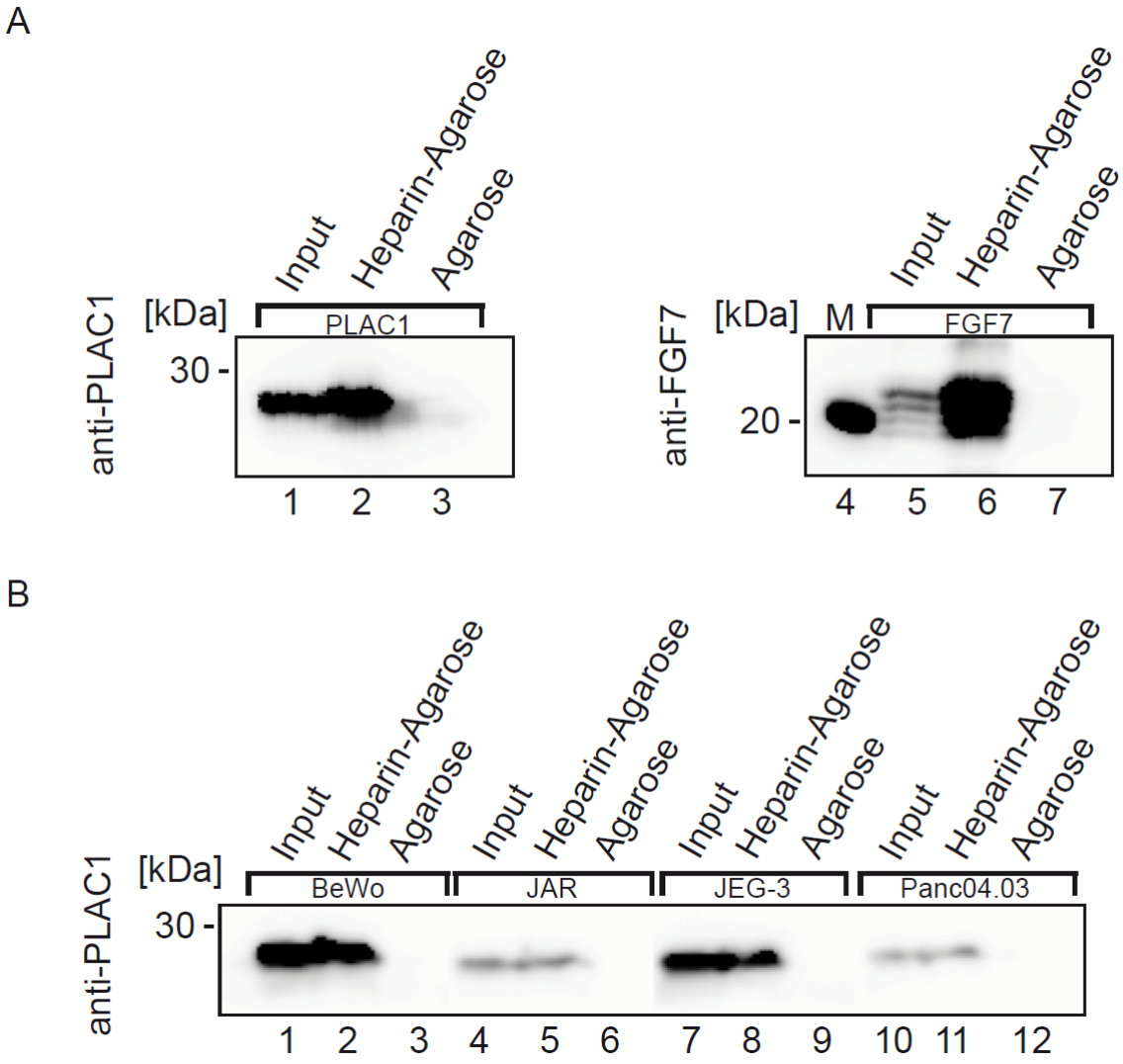

Figure 30. hPLAC1 interacts with heparin.
A. Total cell extracts from HEK293T cells, over expressing human PLAC1 (upper panel, left) or human FGF7 (upper panel, right) were analyzed for binding to Heparin-agarose. As control a free agarose matrix (lane 3 and 7) was used. Lane 1 and 5 show 10% of the used input. Heparin-binding of proteins was assessed by SDS-PAGE and Western blotting with respective antibodies. B. Heparin-binding of endogenous hPLAC1 was analyzed using total extract from different cell lines as indicated (lower panel). Lysat was incubated with Heparin-agarose (lower panel, lane 2, 5, 8, 11) or an agarose control (lane 3, 6, 9, 12). Total input is shown in lane 1, 4, 7, 10. Binding of hPLAC1 to heparin was assessed by immunoblotting with specific PLAC1 antibodies.

3.4.2.1 The placenta specific protein PLAC1 interacts with the polysaccharide heparin via at least two independent binding sites.

Glycosaminoglycans have been described to play important roles in developmental and homeostatic processes. Anyhow, their biological properties in healthy tissue and cancer correspond to their ability to interact with “heparin-binding proteins”. This way heparin acts as a co-factors for growth factor receptor signaling promoting cell proliferation and angiogenesis (114). Considering that hPLAC1 interacts with heparin, the identification of the

hPLAC1 heparin-binding domain was assessed. To analyze the heparin-hPLAC1 binding motive, wild type (aa 1-212) and a set of truncated hPLAC1 variants, which were consecutively shorten by 15 amino acids from the N- or the C-terminus, were used. Binding studies were performed by a radio-immunoprecipitation assay (RIPA). Therefore, WT and truncated proteins were *in vitro* synthesized by a reticulocyte lysate system using radioactive labeled L-[35S] methionine and subjected to a pull down with a heparin cross linked agarose matrix and with a mock agarose matrix. Precipitations were resolved by SDS-PAGE and analyzed by autoradiography (Figure 31). Surprisingly, truncations did not disturb the hPLAC1-heparin binding: all truncated proteins still interact with heparin as shown in Figure 31 (upper panel, lane 1-18).

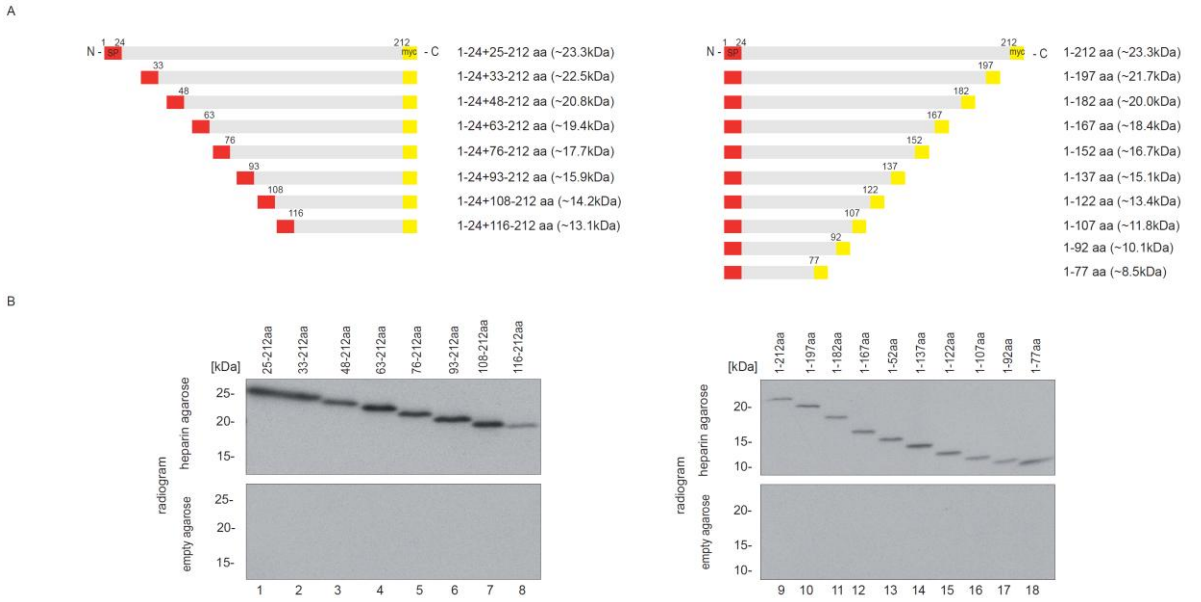

Figure 31. Identification of the heparin binding domain of the hPLAC1 protein.
hPLAC1 WT and successive truncations were *in vitro* translated using the TNT Quick Coupled Transcription/Translation Reticulocyte Lysate System applying radioactive labeled L-[35S] methionine. Proteins were subjected to a pull down with heparin-agarose (upper panels) and empty agarose control (lower panels). Precipitations were resolved by SDS-PAGE and analyzed by autoradiography.

Considering that all constructs share a myc-tag it might be that the myc-tag mediate an unspecific binding to heparin-agarose. Although this phenomenon was not described in the literature, we generated a myc-tagged eGFP to test this possibility. Protein extracts from eGFP and eGFPmyc transfected HEK293T cells were subjected to a heparin pull down (Figure 32). The results obtained after immunoblotting clearly demonstrate that the myc-tag mediates an unspecific binding to heparin, as eGFPmyc (lane 3) but not eGFP (lane 4) binds to heparin.

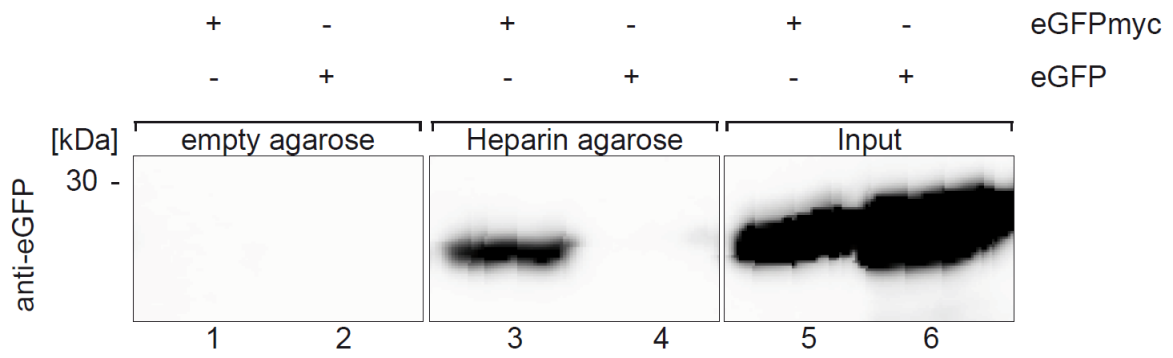

Figure 32. The myc tag mediates an unspecific binding to heparin.
Protein extracts from eGFP and eGFPmyc transfected HEK293T cells were subjected to a heparin pull down (lane 3-4). As control empty sepharose was used (lanes 1-2). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies.

Concluding that, all the tested truncations encode a myc-tag and as shown above the myc-tag itself mediates an unspecific binding to heparin, no information about the heparin binding domain could be achieved. However, to address the question, which domain within hPLAC1 mediated the heparin binding, new constructs were generated as depicted in Figure 33A. These new truncated variants did not encode any tag. Moreover, the sequence encoding the predicted hPLAC1 signal peptide was also deleted since preliminary experiments demonstrated that this region was not essential for heparin binding (data not shown). HEK293T cells were transfected with the respective cDNA and extracts were subjected to pull down experiments and finally analyzed by immunoblotting Figure 33B. hPLAC1 wild type and the truncated variant lacking the predicted signal peptide bind to the same efficiency to heparin (lane 1 and 2). In contrast, proteins coding only for the N- or the C-terminus seem to lose that strong binding efficiency (lane 3 and 4). Nevertheless, a weaker binding was still detectable in both cases, but more intensive within the N-terminus. To summarize these results, hPLAC1 seems to be composed of at least two or more heparin-binding domains within the N- and C-terminus. Anyhow, each of these binding sites is able to weakly interact with heparin. Interestingly, similar results were obtained after the PLAC1-FGF7 domain-mapping (Figure 26-Figure 29). The possibility that both binding partners, hFGF7 and heparin, compete for the same binding site within hPLAC1, will be further discussed.

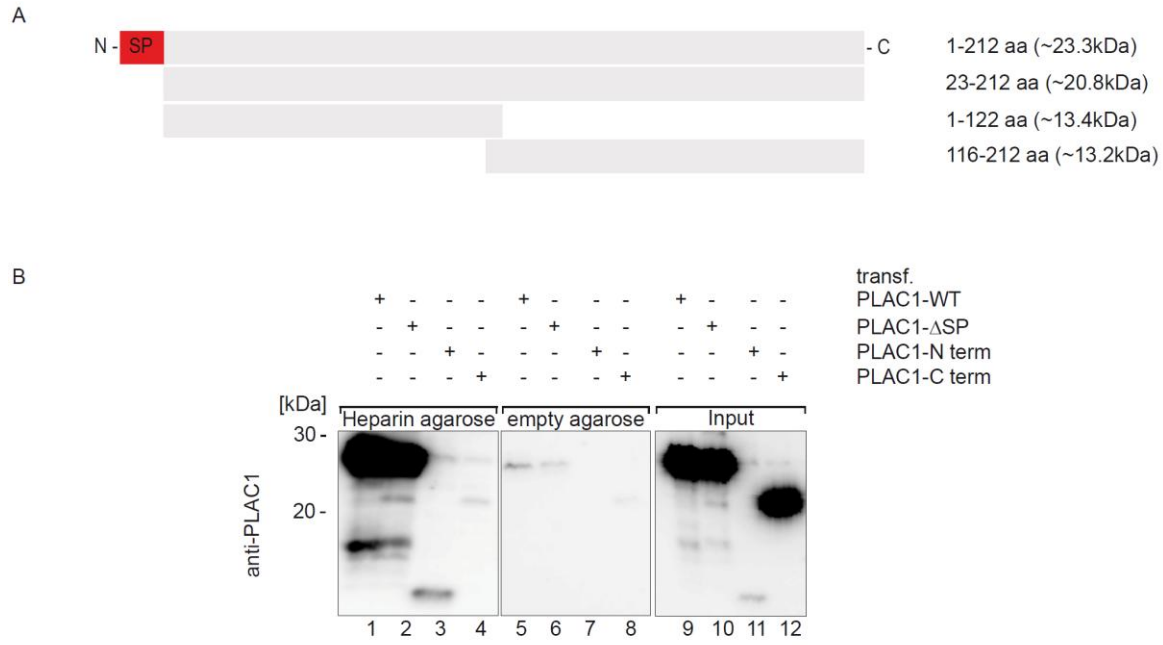

Figure 33. hPLAC1 binds mostly via the N- terminus to heparin.
A. Schematic illustration of different truncations used for the binding studies. The signal peptide is colored in red and hPLAC1 in gray. B. hPLAC1 WT and truncated forms were transfected in HEK293T cells. hPLAC1 was precipitated from extracts with heparin immobilized to sepharose (lane 1-4). As control empty sepharose was used (lanes 9-12). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies.

3.4.3 The placenta specific protein PLAC1 forms a complex with FGF7 and FGFR2IIIb

Fibroblast growth factors mediate their mitogenic effects on target cells by signaling by cell-surface tyrosine kinase receptors, activating various cytoplasmic signal transduction pathways by tyrosine phosphorylation. Crystal structures revealed that the formation of an active complex between FGFs and the FGF-receptors requires the presence of glycosaminoglycans to mediate signaling (115, 116). As mentioned above, the placenta specific protein PLAC1 interacts exclusively with one member of the FGF-family, FGF7. In contrast to other FGFs, FGF7 binds and thereby activates only one receptor isoform, FGFR2IIIb (100, 117). This ligand-receptor interaction has been described to regulate proliferation and stimulate migration in different cancer cell lines. Considering that hPLAC1 regulates proliferation and migration in breast cancer cell lines, evidences were given, that hPLAC1 might be involved in the complex formation of hFGF7, hFGFR2IIIb and glycosaminoglycans. FGF7 and glycosaminoglycans activates the receptor through the extracellular domain, the so called D1-D3 region of the FGFR2IIIb. To this end, only this region was used as a fusion protein for binding studies. The D1-D3 region of the FGFR2IIIb was fused to the Fc-region of an

immunoglobulin. To analyze the complex formation, co-immunoprecipitations were performed. HEK293T cells were transfected with all three components (hPLAC1, hFGF7 and hFGFR2IIIb-IgG). FGF7 was precipitated from the extract by specific antibodies and binding of hPLAC1 or the FGFR2IIIb-fusionprotein was analyzed by Western-blotting. As clearly shown in Figure 34, hPLAC1 forms a complex with the FGFR2IIIb receptor and hFGF7 *in vivo*. Concluding from this result, hPLAC1 might act as a co-factor or activator of the FGF7-FGFR2 signaling pathway and thereby function as interacting partner of this receptor kinase.

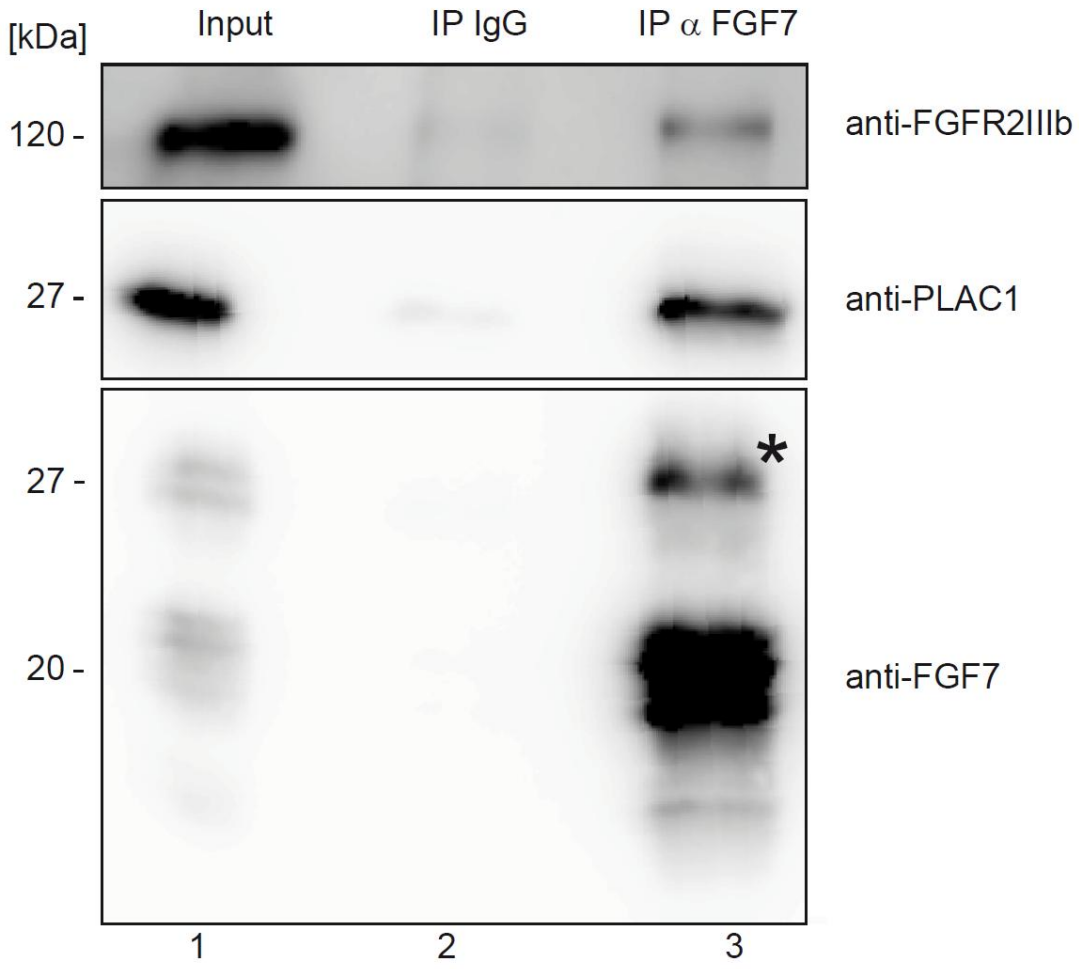

Figure 34. PLAC1 can form a complex with FGF7 and FGFR2IIIb *in vivo*.
hFGF7, hPLAC1 and a rabbit-IgG-FGFR2IIIb (D1-D3) fusion protein were co-transfected in HEK293T cells. hFGF7 was immunoprecipitated from the extract with specific antibodies (lower panel, lane 3), immobilized to NHS-sepharose. As control corresponding, immobilized IgG was used (lane 2). Precipitations were resolved by SDS-PAGE and analyzed by Western blotting with respective antibodies. The input is shown in lane 1. Asterisk represents glycosylated hFGF7 isoforms.

3.5 PLAC1 regulates proliferation of tumor cells

3.5.1 PLAC1 acts as an co-factor of the FGF7-FGFR2 signaling pathway in the breast cancer cell line MCF7

The biological significance of PLAC1 in tumor cells was shown by Koslowski et al. in 2007, demonstrating that siRNA mediated PLAC1 knockdown inhibit cellular proliferation, leading to a G1-S arrest. This arrest was caused by a decreased expression of cyclin D1 and reduced phosphorylation of AKT kinase (Figure 7 & Figure 8). How the AKT-pathway is altered by PLAC1 remains elusive. Concluding from the results presented above and the described data by Koslowski et al. in 2007, it is feasible that hPLAC1 might act as a co-factor or activator of the FGF7-FGFR2 signaling pathway. To confirm this assumption, proliferation rates after siRNA mediated PLAC1 knockdown in dependency of FGF7 were measured. Since the FGFR signaling pathway has been described to be implicated in cancer, especially in breast cancer (118), the PLAC1 expressing breast cancer cell line MCF7 was used in this experimental setup.

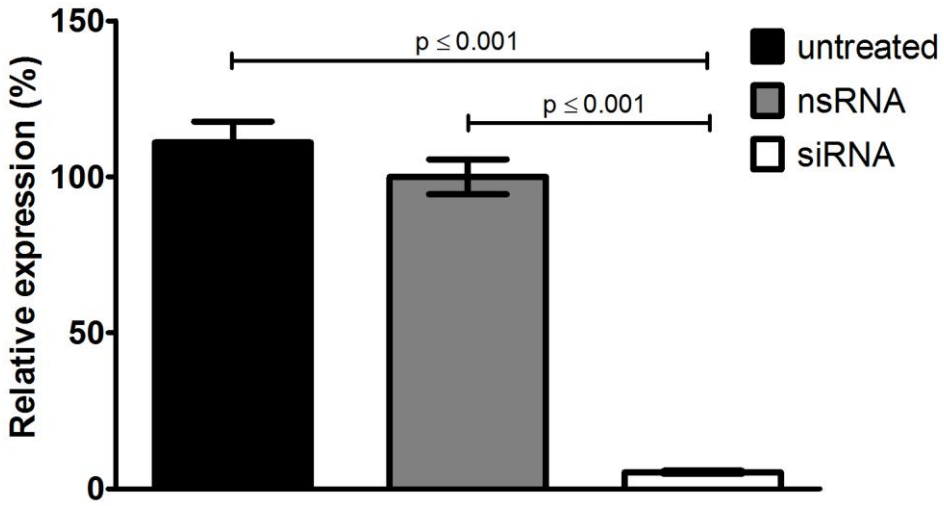

Figure 35. Quantitative real-time PCR analysis of siRNA mediated PLAC1 silencing in MCF7 cells.
MCF7 cells were transfected with a mixture of two different siRNA duplexes (Qiagen) targeting PLAC1 (siRNA 1 targeting nucleotides 342 to 362 and siRNA 4 targeting nucleotides 670 to 690) and as non-silencing control RNA, nsRNA duplex. PLAC1 mRNA expression was analyzed 72h post-transfection by quantitative real-time PCR. Real-time quantitative expression analysis was done in quadruplicates in a 40-cycle qPCR. For normalization the housekeeping gen hypoxanthine phosphoribosyl transferase (HPTR) was used. PLAC1 transcripts of siRNA transfected cells were quantified relative to nsRNA transfected cells using ΔΔCT calculation.

To analyze FGF7 and PLAC1 dependent proliferation, MCF7 cells were cultured at low FCS concentrations (2%). PLAC1 gene silencing was achieved by small interfering RNA (siRNA) transfection. siRNA transfection was carried out with the HiPerFect Transfection Reagent from Qiagen (Germany). To avoid off-target effects, a non-silencing RNA (nsRNA) was used. Knockdown of PLAC1 mRNA expression was analyzed by quantitative real time polymerase chain reaction (qPCR). A stable and reproducible reduction of constitutive PLAC1 mRNA expression by 80-90% compared to controls was achieved (Figure 35). Further, proliferation was analyzed 24h, 48h and 72h after siRNA mediated PLAC1 mRNA silencing. Transfected MCF7 cells were cultured at low FCS concentrations, with or without adding 10ng/ml FGF7 to the media. Proliferation was measured by the incorporation of Bromodeoxyuridin (BrdU), into newly synthesized DNA strands using the DELFIA cell proliferation kit (Perkin-Elmer). The Delfia Cell Proliferation assay is a fluoroimmunoassay so that proliferation was measured on a Wallac Victor2 multi-label counter (Perkin-Elmer). The fluorescence measured in the cell population is proportional to the DNA synthesis of the cells. In Figure 36 proliferation rates of siRNA or nsRNA transfected MCF7 cells cultured at low FCS concentrations with or without FGF7 (10ng/ml FGF7) are shown.

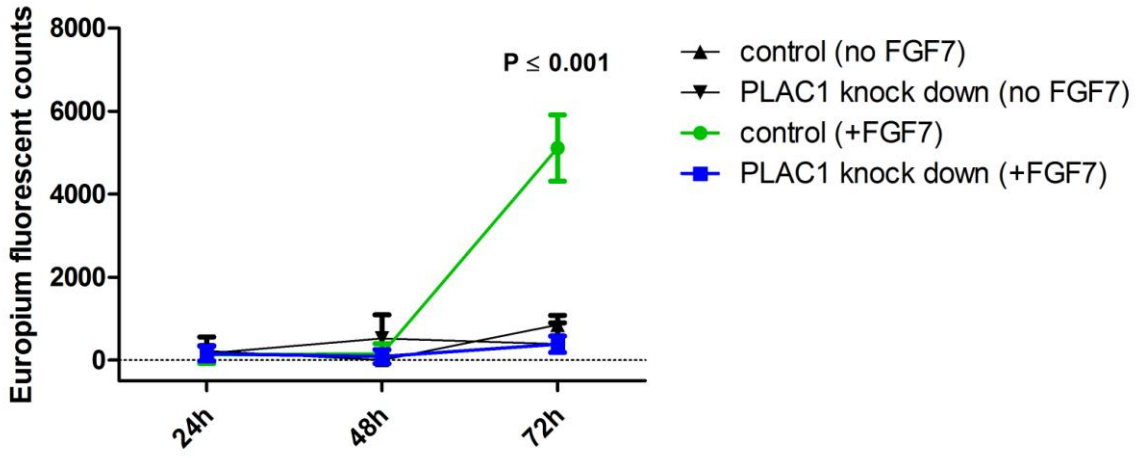

Figure 36. PLAC1 expression promotes the FGF7 mediated proliferation of the breast cancer cell line MCF7.
MCF7 cells were transfected with a mixture of two different PLAC1 specific siRNA duplexes (Qiagen) and a non-silencing control RNA duplex. Cells were cultured at low FCS concentrations with or without FGF7 (0 and 10ng/ml). Proliferation was measured after 24, 48 and 72h using the DELFIA cell proliferation kit.

Even though cells were cultured at very low FCS concentrations and proliferation was generally disturbed, proliferation was inhibited after siRNA mediated PLAC1 knockdown as described by Koslowski et al. in 2007. However, when FGF7 (10ng/ml) was added to the media the effect was strongly increased. PLAC1 silencing cells did not show any BrdU incorporation but MCF7 cells expressing PLAC1 did. Interestingly, proliferation seems to depend on the presents of both proteins: FGF7 and PLAC1. Taken together, these results clearly show that cellular proliferation is dependent on the presents of both proteins -PLAC1 and FGF7- in MCF-7 cells. These and the results described above demonstrated that PLAC1 is a co-factor involved in the FGF7-FGFR2 signaling pathway: PLAC1 promote cell proliferation together with its interacting partner FGF7 by forming a complex with the receptor, stimulating proliferation probably by triggering FGFR2IIIb receptor kinase activity and thereby activating several downstream adaptor proteins i.e. AKT-kinase.

IV DISCUSSION

4.1 Characteristics of the placenta specific protein PLAC1

The placenta specific protein PLAC1 was first described in 2000 by Cocchia and his colleges as an X-linked gene, only expressed in placenta, but not in any other normal human tissue (69). Within the placenta, PLAC1 has a restricted localization at the apical region of the maternal-facing syncytiotrophoblast. PLAC1 was supposed to have an important function during human pregnancy (69, 72). Further it was shown that PLAC1 mRNA expression is influenced by the keratinocyte growth factor (KGF), a factor that regulates normal trophoblast proliferation and differentiation. This observations lead to a model of a placenta-specific PLAC1-KGF-axis, which regulates trophoblast development via paracrine mechanisms (87, 88). However, understanding of PLAC1 function was driven further, when the placenta specific protein PLAC1 was characterized as a new member of the family of cancer-testis antigens, playing a fundamental role in proliferation of cancer derived cell lines (68). At this stage, high expression levels of PLAC1 mRNA were detected in a wide range of human cancers, especially breast cancer, as well as in several cancer cell lines. Further, it was shown that PLAC1 is involved in the regulation of cell cycle progression and consequently cell proliferation. This regulation is driven by the phosphorylation of the AKT kinase (68). The mechanism how PLAC1 expression alters the AKT-pathway remained unclear. Henceforth, PLAC1 and its biological significance in the placenta and in cancer derived cell lines needed to be studied to employ PLAC1 as a new target for cancer immunotherapeutic approaches.

4.2 PLAC1, a new attractive target for immunotherapy?

Immunotherapy is one of the most elegant concepts in cancer therapy. Various strategies, based on recruiting and restoring reactivity of the host´s immune system to combat cancer, were applied. One of these strategies is the antibody-based anti-cancer therapy. Antibody based cancer therapies have been successfully introduced into the clinic and have emerged as the most promising therapeutics in oncology over the last decade. Antibody-based therapies for cancer have the potential of higher specificity and lower side effect profile as compared to

conventional drugs. A prerequisite for the development of such a therapeutical approach is the identification of tumor specific antigens. Targets for antibody-based therapies are, in particular, molecular structures which are exclusively expressed or overexpressed in tumor cells. Moreover, if a molecular structure is functionally involved in cancer development, binding of antibodies to this structure can antagonize its function resulting in a therapeutically beneficial effect. The placenta specific protein PLAC1 is such a tumor specific antigen. Within this work PLAC1 was biochemically characterized and it was analyzed, whether PLAC1 achieves the requirements (mentioned in the introduction; 1.3) to be defined as a highly attractive target for therapeutic antibody approaches.

4.2.1 Protein expression of the placenta specific protein PLAC1 in humans

The first requirement a target structure should comply is to be expressed by a tumor with no or only a restricted expression in tissue. The analysis of the placenta specific protein PLAC1 expression pattern indeed revealed that it is only expressed in placenta and testis, but not in any other normal adult or fetal tissues (72). Furthermore, high expression levels of PLAC1 were observed in a variety of human primary cancers and cancer cell lines (68, 72, 73). However, all these results were obtained by the analysis of the mRNA level of PLAC1 e. g. by northern analysis, *in situ* hybridization and quantitative reverse transcriptase polymerase chain reaction (qPCR). So far tumor associated alterations on protein level of PLAC1 between healthy tissue and tumors were not addressed. For this an exhaustive analysis of PLAC1 protein expression pattern in a high variety of normal human tissue and different human cancer cell lines descending from different organs was performed. Protein expression of PLAC1 was analyzed in man using a set of 22 normal tissue sections that clearly confirmed the described expression of PLAC1 mRNA: Protein expression of PLAC1 is restricted to placenta in man (Figure 9). In contrast to the published qPCR data, no PLAC1 protein expression was detected in testis. Probably the reason therefore is due to much lower PLAC1 mRNA levels and consequently lower PLAC1 protein expression levels in testis than in placenta. This low expression of PLAC1 protein in testis might be too low to reach the detection limit of immunoblotting methods. However, the observed result for PLAC1 expression in testis was not due to the quality of the used samples, since a high quality assurance procedure was given. Independent tissues deriving from five different men revealed the same result on protein levels (data not shown). The protein expression analysis of PLAC1 carried out in different cancer cell lines (Figure 10) demonstrates that there is no different tumor associated migration behavior of PLAC1 in any cancer cell line compared to placenta.

This may indicate that PLAC1 adopts the same function in placenta as in cancer. Moreover, PLAC1 expression was increased in cancer cell lines derived of trophoblastic, breast and pancreatic lineage (Figure 10). These results were consistent with the published transcript analysis, showing PLAC1 expression in breast and trophoblastic cancer cell lines (68, 73). PLAC1 expression in pancreatic cancer cell lines was a very interesting new finding. The feasibility to use PLAC1 as a target for cancer immunotherapy, especially for pancreas as well as for breast cancer patients, underlined the drugability of PLAC1. Particularly when regarding that at least 25% of all cancers worldwide are breast cancer and considering the aggressive nature and the concomitant poor prognosis of pancreatic cancers.

A next step would be to analyze protein expression of PLAC1 in tumor samples of patients or donors and thereby directly compare the protein expression pattern of healthy versus malignant tissue sections in man (breast, pancreas, testis and the corresponding carcinoma). However, this is only realizable via a close collaboration with hospitals and pathologists, who could provide the necessary tissue samples. To obtain clear results, fresh tissues sections as well as well characterized tumor sections are a prerequisite.

Taken together these results, PLAC1 confirm the requirements of a tumor specific protein with no expression in normal tissue beside placenta. Consequently, PLAC1 can be defined as a new target, suitable for antibody-based anti-cancer therapy.

4.2.2 Localization and structur of the placenta specific protein PLAC1

A second requirement a protein should achieve to be defined as a target for antibody mediated immunotherapy, is to be expressed on the surface of tumor cells. In the case of PLAC1 the localization is one of the most controversial discussed findings in the literature, describing PLAC1 as transmembrane protein (68), as membrane-associated protein (74) or as a cytosolic protein with close proximity to the apical plasma membrane (75). However, the criteria describing PLAC1 the localization of PLAC1 are not based on direct experimental data but rely on computer predictions. To clarify the topic of PLAC1 subcellular localization, several biochemical characterization studies were performed. First, the existence of a predicted signal peptide, which conducts PLAC1 to the endoplasmic reticulum (ER) and is cleaved during protein processing, was confirmed (Figure 12). Further, biochemical analysis reveals that PLAC1 was not detectable in the supernatant of cultured PLAC1 expressing cells, indicating that PLAC1 is not a free unbound secreted protein (Figure 13). Finally, subcellular fractionation (Figure 13) as well as immunofluorescence microscopy (data not shown) revealed that PLAC1 is associated to the membrane. This finding was also confirmed by

several approaches implying the localization of PLAC1 at the cell surface e.g. trypsinized BeWo cells compared with untreated cells showed a reduction of PLAC1 expression after trypsin digestion (data not shown). Even though the obtained results clearly demonstrated that PLAC1 is associated to the membrane, the protein did not exhibit a distinct transmembrane protein behavior. On the other hand PLAC1 does also not exist as a free unbound protein, since it was not detectable in the supernatant of cultured PLAC1 expressing cells (Figure 13). This contradiction lead to the hypothesis, that PLAC1 probably polymerizes and thereby forms subramolecular structures outside the cell. Using size exclusion chromatography such high molecular structures, ranking from dimeric to multimeric molecules, could be clearly shown. These subramolecular structures are formed by PLAC1 homodimerization (Figure 17). Especially, the amino-terminal part of PLAC1 is responsible for the formation of high molecular structures (Figure 18). As mentioned in the introduction, a *zona pellucida* (ZP) domain is predicted in the N-terminal part of PLAC1 (69, 76, 81). However, the presence of a *zona pellucida* (ZP) motif has been described to be sufficient for protein polymerization and often lead to the formation of subramolecular structures (76, 81-83). This make it likely that the observed high molecular structures of PLAC1 were formed via the PLAC1-homology region of the ZP domain, as described by Jovine at all (79). Further, a detailed analysis of PLAC1 multimerization and aggregation demonstrate that PLAC1 forms a heterogeneous population of covalent as well as non-covalent monomeric and multimeric forms. Interestingly, these monomeric and multimeric forms are partially resistible to SDS and β-mercaptoethanol treatment. In Figure 37 the different stages of polymerized PLAC1 and their resistance to SDS and β-mercaptoethanol treatment are depicted. Several forms of PLAC1 exist *in vivo*: a monomeric and a multimeric stage of PLAC1 molecules that are interacting non-covalently and a multimeric or aggregated form of PLAC1 that consists of covalent and non-covalent interactions. These aggregates can be well precipitated by ultracentrifugation (1h, 100.000g; data not shown) but are SDS insoluble and irresistible to boiling under reducing conditions. Probably these PLAC1 aggregates were formed after folding and oxidation on their way through the ER and the Golgi and are attached to the surface of the cell, where there build a stable coat-like structure that surrounds the cell. These properties would be in analogy to the properties of the *zona pellucida*: The *zona pellucida* forms a coat that surrounds eukaryotic egg cell. However, the function of PLAC1 within this specialized extracellular coat is unknown. It might provide the exchange of nutrients, wastes and gases between the maternal and fetal system within the placenta or within the tumor and thereby function like a nest for mitogens or other stimulating factors.

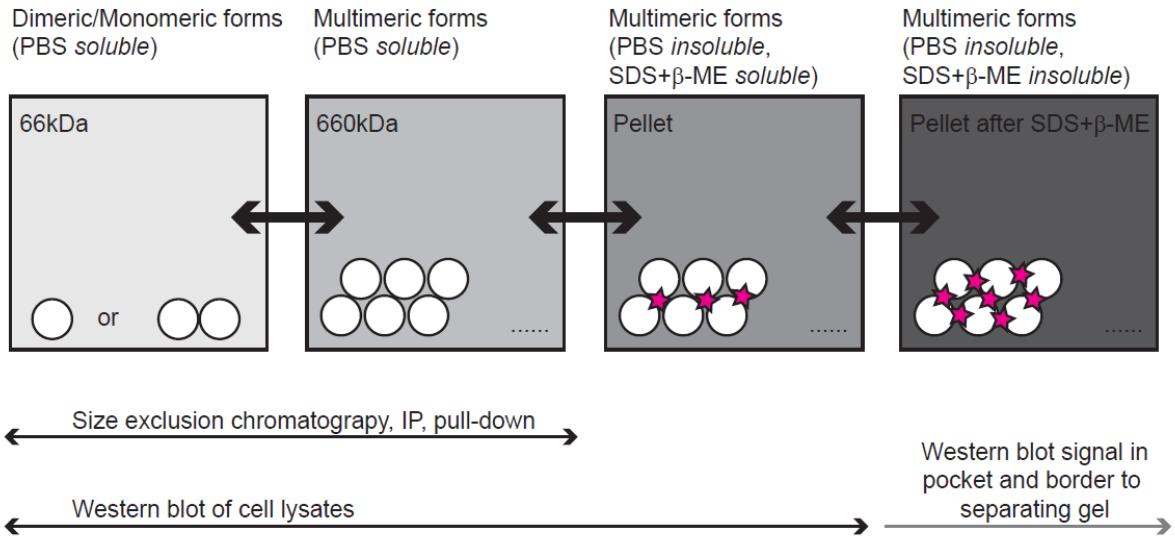

Figure 37. Nature of PLAC1 aggregation and multimerization.
Several forms of PLAC1 exists *in vivo*: a monomeric and multimeric form that is soluble in a PBS-buffer, a multimeric form that is insoluble in PBS-buffer but solubilized by a SDS-buffer containing β-mercaptoethanol and finally a multimeric structure that is also resistant and therefore insoluble in SDS-buffer containing β-mercaptoethanol.

How does this knowledge influence the proposal to use PLAC1 as a target for anti-cancer therapy? Since there are several approaches indicating that PLAC1 localize at the surface of the cell, the development of a monoclonal antibody (mAb) seems obvious. The first criterion the mAb should achieve is to bind and label cells that express the antigen on their surface. Through the constant part of the antibody the complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) gets induced until cytotoxic effector cells lyze the antibody labeled tumor cells. Several immunotherapeutica induce CDC or ADCC (e.g. Rituximab). However, therefore the availability of the antigen for the mAb must be ensured. Based on the multimerization of PLAC1 and its hypothetic random-coiled structure, the availability of a specific sequence targeted by a monoclonal antibody seams difficult. Moreover, the available antibodies targeting PLAC1 did not show an ADCC or CDC effect (data not shown). Considering this and the status PLAC1 adopts on the surface of the tumor cell, the combination of several PLAC1 specific antibodies would lead to a better targeting of the multimeric PLAC1 population. Interestingly, a mixture of several mAb targeting divers epitopes located at different domains, e.g. at the N- or C-terminus of the PLAC1 protein, was already tested and revealed much better targeting and precipitation of endogenous and over expressed PLAC1 (data not shown).

In summary, the trophoblast specific protein PLAC1 contains a functional secretion signal and is stacking to the extracellular matrix after it is exported from the cell. Outside the cell PLAC1 forms a complex structural entity by multimerization. The observed high molecular multimers and oligomers consist of a mixture of non-covalent as well as covalent interactions. Thereby PLAC1 interact with itself and probably other cellular components and proteins and form a strongly connected and stable multi-protein complex leading to a specialized extracellular matrix. Consequently, this new type of extracellular localization and aggregation of PLAC1 has important implications for the drugability of PLAC1. However, since PLAC1 display relevance during malignant phenotype development, it gets more interesting for the development of monoclonal antibodies that disturb or disrupt the function of the target molecule. These antibodies were designed to act on cellular and molecular targets that are causally involved in the formation, growth, and progression of human cancers e.g. Trastuzumab (Herceptin). Therefore, it is necessary to have an understanding of the underlying molecular functions and mechanisms the target is involved.

4.2.3 Analyzing the interacting network of PLAC1

One of the most important characteristics a drug target should exhibit is to be involved in a cellular process during tumorgenesis. The placenta specific protein PLAC1 exhibit such a cellular function. It was shown that PLAC1 is involved in the regulation of cell cycle progression and consequently in cell proliferation of breast cancer cell lines. Further, this regulation is driven by the phosphorylation of the AKT kinase (68). But the pathway leading to the activation of AKT kinases remained unclear and was addressed within this work.

The first hint how PLAC1 alters the AKT kinase was achieved by identifying the fibroblast growth factor FGF7 as a specific interacting partner of PLAC1 (Figure 19). Several members of the FGF-family were analyzed but only FGF7 binds to PLAC1. This result was very surprising, since the FGF-family consist of 23 members that are highly homologous as shown in Figure 20 (99). Moreover, the interaction between PLAC1 and FGF7 was conserved in different species as shown in Figure 23. These results underlined the specificity and importance of this interaction within a cellular process. So far, the FGF signaling pathway has been characterized to play a significant role in normal development and wound healing but also in tumor development and progression, where it regulates cell proliferation, differentiation and motility (100-102, 119). Specially FGF7 has been described to exhibit mitogenic functions and has a significant expression in estradiol-receptor positive breast- and pancreatic- carcinoma cell lines (103-106, 120-124). Interestingly, PLAC1 was also

overexpressed by breast- and pancreatic- carcinoma cell lines (Figure 10). Moreover, FGF7 was described to belong to the MMTV target oncogenes. MMTV, mouse mammary tumor virus, is a retrovirus that acts as an insertion mutagen by integrating into the host genome and perturbing host gene expression. MMTV integration leads to the activation of different oncogenes that belongs to the FGF-, to the WNT- or the NOTCH- family. However, several of these MMTV target oncogenes are significantly deregulated in a subset of human breast cancers (125, 126). Specially in the case of FGF7 and its receptor FGFR2, it was shown that FGF7 as well as FGFR2 expression alters epithelial cell proliferation in human breast cancer (106, 127) and that its constitutively overexpression in the mammary epithelium of transgenic mice lead to an induction of mammary carcinoma (118). Considering that PLAC1 expression is also altered in mammary carcinoma and a direct interaction between PLAC1 and FGF7 was observed, a functional relevance which probably results in an activation of a cellular process was supposed. Interestingly, FGF7 was already identified to have a regulative function concerning the expression of PLAC1: the level of PLAC1 mRNA increased upon treatment of cells with FGF7 (72). Probably the deregulation of the MMTV target oncogene FGF7 occurs as an initiating step in the development of breast cancer and induces in a following step the expression of PLAC1. The interaction between PLAC1 and FGF7 would then lead to an alteration of proliferation and motility, probably due to the induction of the FGF signaling pathway. Interestingly, PLAC1 also exhibits a strong affinity to heparin (Figure 30) and heparan sulfates (data not shown). Heparan sulfate (HS) and heparin, which were highly similar in structure, were described to bind to FGFs and FGFRs, facilitating the formation of a ternary complex consisting of two heparin moieties, two FGF molecules and two FGFRs (112, 113). Within this complex heparin promote the dimerization of the ligand-receptor complex, whereby FGFs mediate their cellular responses by activating the intracellular receptor tyrosine kinase via its phosphorylation in trans (100-102). However, it is supposed that heparin primarily serves only to dimerize FGFs and juxtapose other components of the FGF signaling-transduction pathway. Considering this and that PLAC1 bind to heparin, it might be that PLAC1 gets somehow placed by heparin in the right position within the ligand-receptor complex. Interestingly, several evidences imply that PLAC1 directly or indirectly modulate the biological activity of FGF7 or its receptor. Four high-affinity receptors (FGFR1-4) are described within the literature. By alternative-splicing mechanism several FGFR isoforms were generated. These isoforms display specific ligand binding properties. Since PLAC1 exclusively interacts with FGF7 only the FGF7 specific receptor was analyzed. FGF7 bind to a specific FGFR2 splicing isoform, FGFR2IIIb. Within this work it was shown that

PLAC1 together with FGF7 and the receptor FGFR2IIIb form a trimeric complex (Figure 34). A direct interaction of PLAC1 with the receptor was not observed (data not shown). PLAC1 would exhibit its function acting as a co-factor, which modulates the FGF-signaling pathway by interacting directly with FGF7 and thereby indirectly with the receptor. But how can these new data be related to the known functional characteristics of PLAC1? In 2007 Koslowski et al. demonstrated that siRNA mediated PLAC1 knockdown inhibits cellular proliferation, leading to a G1-S arrest. This arrest was caused by a reduced phosphorylation of AKT kinase which results in a decreased expression of cyclin D1 (Figure 7 & Figure 8). Interestingly, the AKT pathway is crucial to many aspects of cell proliferation and survival (128, 129). How PLAC1 alters the AKT pathway was clarified by the findings described in this work. The first hint how PLAC1 alters AKT kinases was achieved by identifying the fibroblast growth factor FGF7 and heparin as specific interacting partners und demonstrating that PLAC1 is involved in the formation of an FGF-ligand receptor complex. The FGF signaling pathway has been characterized to regulate cell proliferation, differentiation and motility (100-102). FGF mediated cell proliferation includes also the activation of the signal node proteins AKT. Since PLAC1 as well as FGF7 seems to be involved in cell proliferation, proliferation rates after siRNA mediated PLAC1 knockdown were measured in dependency of FGF7. Interestingly, proliferation depends on the presents of both proteins: FGF7 and PLAC1 (Figure 36). Considering these data, it seems that PLAC1 functions by stimulating proliferation by acting as a co-factor of the FGF7-FGFR2 signaling pathway. However, the conclusive proof that PLAC1 alters as a co-factor the FGF7-FGFR2 signaling pathway was achieved using the PathScan RTK Signaling Antibody Array Kit from Cell Signaling Technology and the Human Phospho-RTK Array Kit from R&D Systems. It was clearly shown, that PLAC1 activates the phosphorylation of AKT as well as MAPK by specifically stimulating and activating the FGFR2IIIb receptor (Figure 38 A-B, kind regards to Dr. Christoph Hartmann and Dr. Stephanie Hubich). In addition we observed that the FGF7 induced phosphorylation of AKT is reduced in PLAC1 knock down cells (k.d.) compared to control cells (control). Interestingly, these findings were observed in BeWo cells, a choriocarcinoma cell line, and several breast cancer cell lines (SkBr3-, T47D- and MCF7- cells) (Figure 38 C-D).

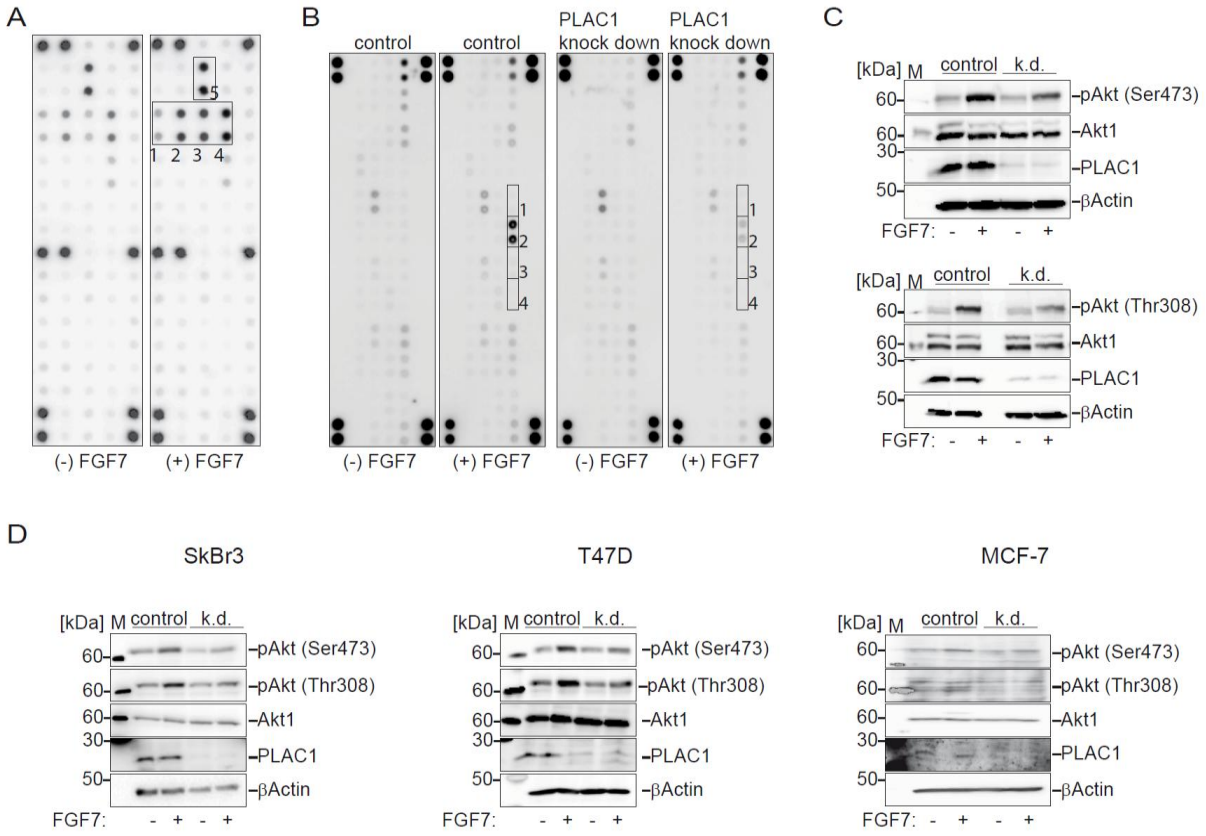

Figure 38. PLAC1 activates AKT phosphorylation by specific stimulation and activation of FGFR2IIIb receptor.
(A) The FGF7 stimulation of BeWo cells leads to a specific phosphorylation of the signal node proteins AKT (on Thr308 (1) and Ser473 (2)) as well as MAPK (3). We also see activation of S6 (4) and Src (5) protein. (B) Only the FGFR2 receptor is activated by FGF7 (2) in contrast to FGFR1 (1), FGFR (3) and FGFR (4) (control, left side). In PLAC1 knock down cells (k.d, right side) these FGF7-specific FGFR2 activation is blocked. (C and (D) The stimulation of AKT is influenced by PLAC1 in (C) BeWo Cells and (D) several breast cancer cell lines. The FGF7 induced phosphorylation of AKT Ser473 (upper panel) is reduced in PLAC1 knock down cells (k.d.) compared to control cells (control). The same effect is detectable for AKT Thr308 (lower panel, k.d. vs. control). For internal control AKT1, PLAC1 and β-Actin was detected with specific antibodies.

The participation of PLAC1 in the FGF-signaling pathway leads to new challenges using PLAC1 as a target for antibody mediated anti-cancer therapy. Above, PLAC1 was described to be a potential antigen for the development of an antibody. Considering that PLAC1 promote cell proliferation together with its interacting partner FGF7 and heparin by forming a complex with the FGFR2IIIb receptor, the generation of a therapeutic mAbs got even more interesting. As described above mAb can serve as antagonists or agonists. Based on PLAC1, a mAb targeting PLAC1 should lead to an inhibition of proliferation, blocking the formation of the PLAC1/FGF7/Heparin/FGFR2IIIb complex. One of the most popular immunotherapeutica in cancer therapy is Herceptin (Trastuzumab). Trastuzumab has been designed to attach to the human epidermal growth factor receptor 2 (HER2). HER2, also known as HER2/neu, is a receptor tyrosine kinase that activates several downstream signaling cascades leading to proliferation, migration and survival. The antitumor activity of

Trastuzumab is attributable to several mechanisms: first Trastuzumab attaches to HER2/neu and thereby block downstream signaling and second Trastuzumab induces ADCC and CDC. In contrast to HER2/neu, which is overexpressed in 20-25% breast cancers, more than 80% of breast cancers overexpress PLAC1 (68, 130). Considering that, more breast cancer patients could be treated with a mAb targeting PLAC1 than targeting HER2/neu. As mentioned above, a therapeutical mAb targeting PLAC1 should inhibit the PLAC1-FGF7 or the PLAC1-heparin mediated signaling. Perturbation of the interaction by an antibody could inhibit the potential mitogenic effects of FGF7. Therefore the specific interacting domain of FGF7 or heparin with PLAC1 was determined in this work. Mapping of the hFGF7 binding domain within the PLAC1 protein was analyzed applying wild type and various N- and C- terminal truncated as well as intermediate versions of PLAC1 which were subjected to several binding assays. The results presented above clearly indicate that PLAC1 possesses at least two or more FGF7 binding sites within its N- and C-terminus. Interestingly, the availability of a signal peptide (aa 1-24) was crucial for the PLAC1-FGF7 interaction, but was not involved in the PLAC1-FGF7 interaction by its own (Figure 27), since fusion of the amino acids 1-24 of PLAC1 to eGFP did not lead to an interaction with FGF7 (data not shown). Concluding, the export of PLAC1, which is mediated by its signal peptide (aa1-24), via ER and Golgi is a prerequisite for its interaction with FGF7. Moreover, PLAC1 exhibits several FGF7 binding sites which are able to interact independently with FGF7. These findings lead to the assumption that PLAC1 probably acquires a special folding or some modification which were essential for the interaction with FGF7. Probably protein folding of PLAC1 leads to a formation of a pocket where N- and C-terminus of PLAC1 gets in spatial proximity and trap FGF7 like a tweezers. Interestingly, two molecules of FGF7 contact each FGFR2IIIb (131). However, PLAC1 might not only be important to get the components in close proximity to the receptor, but also for the conformational relationship of the components towards the formation of the complex. However this is only a model how PLAC1 interacts with FGF7. To verify this hypothesis it would be useful to analyze the protein structure of PLAC1 or to resolve the structure of the PLAC1-FGF7 complex by nuclear magnetic resonance spectroscopy (NMR spectroscopy). Since PLAC1 also interacts with heparin, a glycosaminoglycan (GAG) that is also involved in FGF-signaling, the specific interacting domain of heparin within the PLAC1 protein was determined as well. Mapping of the heparin binding domain within the PLAC1 protein was analyzed applying wild type as well as various N- and C- terminal truncated versions of PLAC1 which were subjected to several binding assays. Unfortunately all the tested N- and C- terminal truncated versions of PLAC1 bear a myc-tag that mediates an unspecific binding

to heparin (Figure 32). This result was very confusing, since heparin as well as the myc-tag is negatively charged and should not mediate an unspecific interaction. Moreover, there is no literature describing such an intrinsic binding between heparin and the c-Myc. To the contrary, myc-tagged proteins are often used to analyze its binding to heparin (132, 133). However, untagged N- and C- terminal truncated variants of PLAC1 were subjected to several heparin binding assays. Wild type and the truncated variant of PLAC1 lacking the predicted signal peptide bind to the same efficiency to heparin. In contrast, proteins coding only for the N- or the C-terminus seem to lose that strong binding efficiency, but still weakly interact with heparin. Interestingly, PLAC1 seems to be composed of at least two or more heparin-binding domains within the N- and the C-terminus, similar to the results obtained after the PLAC1-FGF7 domain-mapping (Figure 26-Figure 29). Probably the binding region of both binding partners, FGF7 and heparin, are in close proximity within the PLAC1 protein or both interacting partners, FGF7 and heparin, compete for the same binding site within PLAC1. Regarding that heparin is also involved in receptor activation as well as FGF7, a competition of FGF7 and heparin for the same binding side within the PLAC1 protein would be inconsequent. Probably the interaction of PLAC1 with heparin is due to facilitate the formation of the FGF/PLAC1 signaling complex.

How do this PLAC1-FGF7 and this PLAC1-heparin binding study influence the development of a PLAC1 targeting antibody? As mentioned above a therapeutical mAbs targeting PLAC1 should lead to an inhibition of proliferation, blocking the formation of the PLAC1/FGF7/FGFR2IIIb complex. Since PLAC1 seems to have at least two FGF7 as well as two heparin binding domains, which independently bind to FGF7 or heparin, it would be necessary to block both sides by specific antibodies. The perturbation could be achieved using a mixture of several mAb targeting both domains within PLAC1. Using a mixture of several mAb targeting different epitopes could also be used to target the specialized extracellular matrix, formed by strongly connected and stable PLAC1 multimers. In this case not only the interaction between PLAC1 and FGF7 or heparin could be perturbed, even cells expressing PLAC1 at their surface could be targeted.

V CONCLUSIONS - Model of the PLAC1 mediated FGF-signaling

The placenta specific protein PLAC1 was proposed to be a highly promising target for immunotherapeutical approaches, but the molecular function of PLAC1in cancer was elusive. Within the present study it was clearly shown that PLAC1 alters proliferation of cancer cells by the FGF signaling pathway. The outcome of this study results in a model depicted in Figure 39: (A) secreted PLAC1 forms strongly connected and stable multimers at the cell surface. These PLAC1 multimers develop a specialized extracellular coat together with the components of the normal extracellular matrix (ECM) (e.g. glycosaminoglycans (GAGs)). (B) This extracellular coat attracts and traps mitogens: especially secreted FGF7 strongly binds to PLAC1. (C) Interacting PLAC1-FGF7 molecules were released from this network and bind to the receptor, FGFR2IIIb. (D) Binding of the PLAC1-FGF7 complex together with a glycosaminoglycans moiety promotes the dimerization of the receptor. (E) Dimerization of the FGFR2IIIb-PLAC1-FGF7-complex activates the intracellular tyrosine kinase of the FGFR2IIIb-receptor by causing its phosphorylation in trans. (F) Further, the active kinase mediates signal transduction through the direct phosphorylation of adaptor proteins as well as by phosphorylating sites on the receptor that act as docking sites for other effector proteins (100-102, 132). The most prominent adapter protein is phosphatidylinositol-3-kinase (PI3Ks). Upon activation PI3K leads to the generation and buildup of PIP3 (Phosphatidylinositol (3,4,5)-triphosphate), which results in the activation of PDK1 (3-phosphoinositide-dependent protein kinase-1). Activated PDK1 recruits and activates AKT. AKT, also referred to as PKB, play a critical role in multiple cellular processes such as cell survival, cell proliferation and cell migration (133, 134). Consequently, our data and data published by Koslowski et al. 2007 are in agreement that PLAC1 alters the phosphorylation of AKT by activating the FGF7/FGFR2IIIb signal transduction pathway and thereby regulate proliferation.

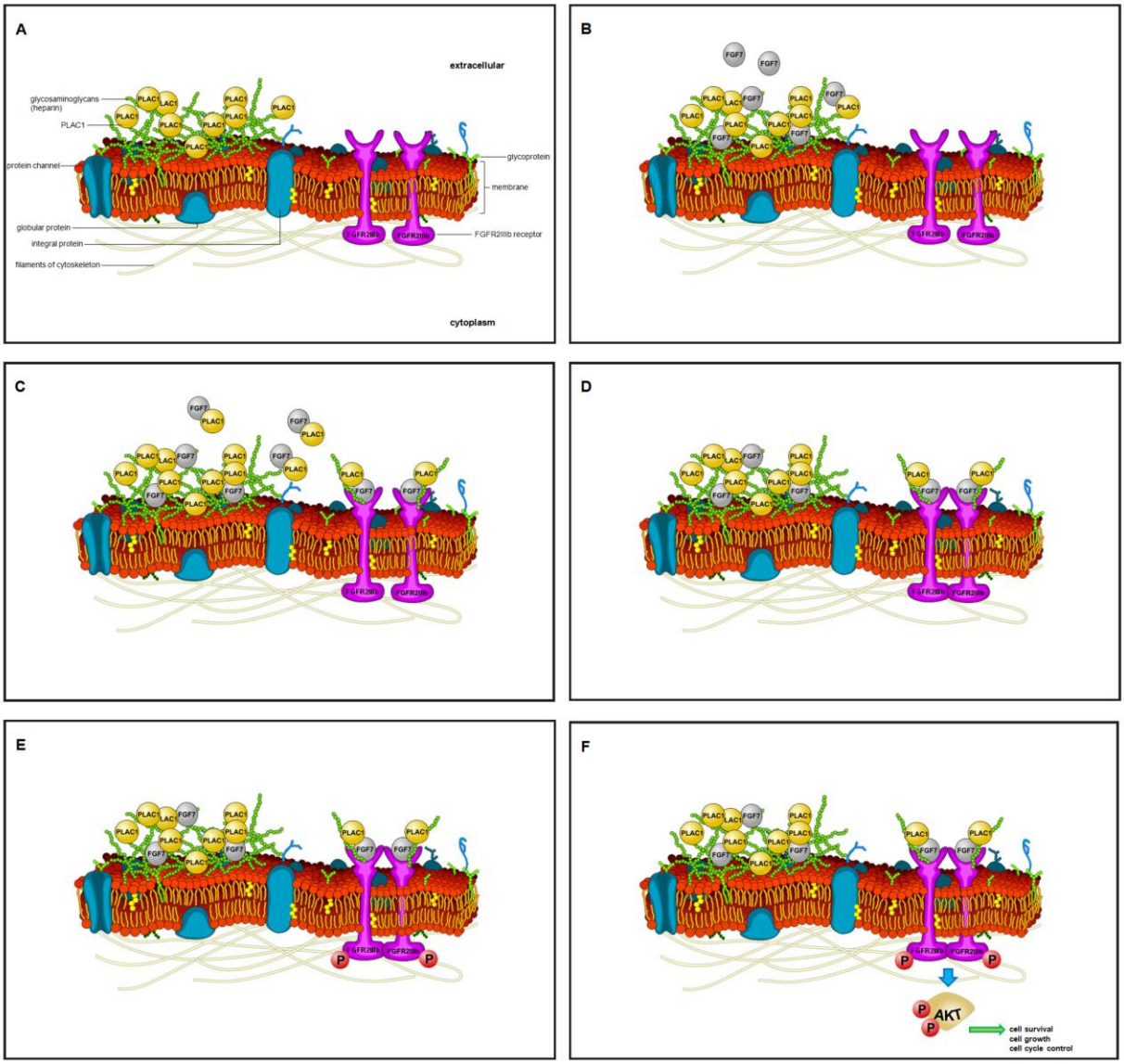

Figure 39. Model of the PLAC1 mediated FGF-signaling.

(A) Secreted PLAC1 forms a strongly connected and stable multimers at the cell surface. These PLAC1 multimers interact with components of the extracellular matrix (ECM) (e.g. heparin). (B) Paracrine secreted FGF7 binds to the ECM, because of its strong affinity for GAGs and PLAC1. (C) Interacting PLAC1-FGF7 molecules were released from the ECM and bind to the receptor, FGFR2IIIb. (D) Binding of the PLAC1-FGF7 complex together with the glycosaminoglycan moiety promotes the dimerization of the receptor. (E) Dimerization of the FGFR2IIIb-PLAC1-FGF7-complex activates the intracellular tyrosine kinase of the FGFR2IIIb-receptor by causing its phosphorylation in trans. (F) The active kinase mediates signal transduction through the direct phosphorylation of adaptor proteins as well as by phosphorylating sites on the receptor that act as docking sites for other adaptor proteins. (100-102). Activated adapter proteins leads to the recruitment and activation of AKT. AKT play a critical role in multiple cellular processes such as cell survival, cell proliferation and cell migration by binding and regulating several targets (133, 134).

Even the role of PLAC1 within the FGF7/FGFR2 signaling pathway was clarified, the specific molecular function remains elusive.

Probably, PLAC1 adopt an important function concerning the attractants of FGF7 in cancer? The distribution of cells expressing FGF7 and its receptor (FGFR2IIIb) clearly demonstrated that FGF7 is a paracrine effector: FGF7 is expressed by mesenchymal cells but mediate proliferation and migration of epithelial cell expressing the receptor (FGFR2IIIb) (105, 134). The above described specialized extracellular coat-like structure, formed by strongly connected and stable PLAC1 multimers, could provide a better surface to attract paracrine FGF7. Further, PLAC1 would not only function as a co-factor regarding the FGF-signaling pathway, but also be involved in the generation of a tumor microenvironment that binds FGF7. Moreover, it is known that FGF7 binds only with moderate affinity to its receptor (K_D: $1.04x10^{-5}$) (135). Considering the low affinity FGF7 exhibit towards FGFR2IIIb, it might be possible, that PLAC1 strengthen and thereby extend the binding of FGF7 to its cognate receptor, leading a stronger activation and thereby to higher proliferation rates. To further strength the finding, that PLAC1 increases the FGF7 induced phosphorylation of FGFR2IIIb and AKT and thereby lead to a stronger cellular signaling, it would be beneficial to analyze, if dissociation rates (K_D) of FGF7 from the receptor (FGFR2IIIb) change in dependency of PLAC1. Besides, the function of PLAC1 was clarified: PLAC1 alters proliferation activating the FGF-signaling pathway. These findings were obtained using BeWo cells, a choriocarcinoma cell line and several breast cancer cell lines (SkBr3-, T47D- and MCF7-cells). To prove that PLAC1 is a universal factor that modulates tumor growth and thereby promotes tumor progression, it would be necessary to analyze the role of PLAC1 in other tumor identities (e.g. pancreas cancer) and to check whether PLAC1 alters tumor progression always by the same mechanism in various cancer cell lines.

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PUBLICATIONS

Articles

Barea Roldán D, Grimmler M, Hubich S, Hartmann C, Cagna G, Rohde C, Koslowski M, Huber C, Türeci Ö & Sahin U. The placenta specific protein PLAC1 promotes tumor progression by stimulating and activating the FGF7-FGFR2IIIb mediated pathway.
Manuscript is in preparation.

Posters

Barea Roldán D, Grimmler M, Hubich S, Hartmann C, Cagna G, Rohde C, Koslowski M, Huber C, Türeci Ö & Sahin U. The placenta specific protein PLAC1 influence cell proliferation by interacting with FGF7 and its receptor FGFR2IIIb *11th Annual Cancer Immunotherapy (CIMT) Meeting* (2011)

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