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CD82, but not CD63, is linked to cellular invasiveness in human thyroid carcinoma

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Referat und bibliographische Beschreibung

In der vorliegenden experimentellen Arbeit wurde die Bedeutung der Tetraspanine CD82 und CD63 (transmembrane-4-superfamily -TM4SF) für das Wachstum der menschlichen Schilddrüsenkarzinome und für die Ausbildung von Metastasen untersucht.
In verschiedenen soliden Tumoren des Menschen spielt CD82 eine wichtige Rolle in den Prozessen der Zelldifferenzierung, der Apoptose, der Zelladhäsion sowie der Metastasierung. Es ist bekannt, dass CD82 in Prostatakarzinomen die Ausbildung von Metastasen unterdrückt, dass CD63 die Motilität maligner Melanomzellen reguliert und in die Prozesse der Zelladhäsion involviert ist.

Die mRNA- und die Proteinexpression der Tetraspanine CD82 und CD63 wurde mit Hilfe der RT-PCR und der Immunhistochemie in 75 malignen Schilddrüsengeweben (24 FTC, 33 PTC, 18 UTC) sowie in 12 benignen Veränderungen der Schilddrüse (Struma colloides et nodosa) bestimmt.
In den untersuchten benignen Schilddrüsengeweben wurde CD82 sowohl auf der Ebene der mRNA, als auch auf der Ebene der Proteine hoch exprimiert und in den nicht metastasierten Schilddrüsenkarzinomgeweben stark oder mäßig (66,7 % auf mRNA Ebene, 58 % auf Proteinebene). Die metastasierten Schilddrüsenkarzinomgewebe zeigten dagegen nur eine schwache oder keine Expression des Tetraspanins CD82. Die Unterschiede in der Expression zwischen den verschiedenen Tumorentitäten der Schilddrüse und den Metastasen waren statistisch signifikant ($p<0,05$). In Bezug auf die Tumorgröße, die Lymphknotenbeteiligung und die hämatogene Metastasierung waren Korrelationen mit dem Expressionsgrad von CD82 feststellbar. Es bestand eine signifikante Korrelation zwischen der CD82 Expression und dem Tumorstadium.
Das Tetraspanin CD63 wurde in allen malignen und benignen Geweben der Schilddrüse nachgewiesen. Die follikulären Schilddrüsenkarzinome zeigten eine höhere Expression an CD63 als die benignen Gewebe, diese korrelierte aber nicht mit dem Tumorstadium.

Eine stabile Transfektion mit dem Ziel der „Überexpression“ von CD82 in FTC-133 Zellen wurde mit einem eukaryotischen Expressionskonstrukt (pcDNA3.1) für CD82 erreicht (pcDNA3.1-CD82). Die Ergebnisse der Untersuchungen mit dem MTT-Vitalitätstest und der „Boyden Chamber“ Test zeigten eine signifikant geringere Rate der Zellproliferation und eine geringere Zellmotilität nach Überexpression von CD82 in der follikulären Schildrüsenkarzinom-Zelllinie FTC-133.

Mit Hilfe der 2D Technologie wurden die Profile der Gesamtproteine der Wildtyp FTC-133, FTC-133-mock und der FTC-133-CD82 Transfektanten im Polyacrylamidegel nach den unterschiedlichen isoelektrischen Punkten (pI) und Molekulargewichten aufgetrennt. 46 verschiedene Proteinspots wurden in den Extrakten der Wildtyp FTC-133, der FTC-133-mock und der FTC-133-CD82 Transfektanten mittels Massenspektrometrie (MALDI-TOF/Q-TOF) identifiziert, die sehr wahrscheinlich mit den Prozessen der Zelldifferenzierung, der Apoptose, der Zelladhäsion sowie der Ausbildung von Metastasen assoziiert sind.

Zusammenfassend kann postuliert werden, dass CD82 ein Marker für die Malignität und die Progenose der Schilddrüsenkarzinome des Menschen ist. Die CD82 Expression in fortgeschritten Schilddrüsenkarzinomen wird herunterreguliert und korreliert mit dem Tumorstadium, insbesondere mit der Ausbildung von Metastasen. CD63 zeigt keine Korrelationen mit den verschiedenen klinischen sowie pathologischen Parametern der untersuchten Schilddrüsengewebe. Die Funktionen der Tetraspanine CD82 und CD63 in Schilddrüsenkarzinomen sollte auch weiterhin untersucht werden.

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Abstract

In this study we investigated the roles of two members of Tetraspans transmembrane family (TM4SF) CD82 and CD63 and their involvement in the process of tumor growth and metastasis in human thyroid carcinoma. CD82 serves as a tumor-metastasis-suppressor in human prostate carcinoma and in many other human solid tumors. CD82 plays an important role in cell differentiation, apoptosis, cell adhesion and metastasis. CD63 plays a critical role in the regulation of cell motility of human malignant melanoma and is also involved in cell adhesion.

Employing RT-PCR and immunohistochemistry, mRNA and protein expression of CD82 and CD63 were investigated in 75 malignant neoplastic thyroid and 12 benign goiter tissues (24 follicular thyroid carcinoma, 33 papillary thyroid carcinoma, 18 undifferentiated thyroid carcinoma and 12 goiters). CD82 was strongly expressed in all investigated benign goiter tissues. CD82 was present strong to moderate 66,7% at the mRNA level, 58% at the protein level, respectively in primary thyroid carcinoma and weak to negative 60,7% at the mRNA level, 82% at the protein level, respectively in metastatic thyroid carcinoma ($p<0,05$). The expression of CD82 in thyroid carcinoma was significantly correlated with the tumor staging. On the other hand, CD63 expression was found in all benign and malignant thyroid tissues. CD63 expression pattern did not correlate with tumor stages.

We generated stable CD82-overexpression follicular thyroid carcinoma cell transfectants (FTC-133-CD82) to study the protential role of CD82 on proliferation and migration of FTC-133. The results from MTT- and Boyden chamber assays demonstrated that the overexpression of CD82 caused a decrease in both cellular proliferation and motility.

Preliminary proteomic analysis of wild type FTC-133, FTC-133-mock and FTC-133-CD82 transfectants was performed using 2D electrophoresis and proteins were separated according to their different isoelectric points “pI” values and molecular weight values. Fourty-six protein spots were identified by mass spectrometry (MALDI-TOF/Q-TOF). Many of the regulated proteins were closely associated with processes of cell differentiation, apoptosis, cell adhesion and metastasis.

In summary, down-regulation and loss of CD82 expression was found in clinically advanced human thyroid carcinomas. Also, human thyroid carcinoma metastases displayed significantly decreased levels of CD82. We postulate that CD82 is a malignancy/prognosis marker for human thyroid carcinoma and affects the tumorigenic potential of human thyroid carcinoma cells. CD63 expression was not correlated with any clinical pathological parameter and the role of this factor in human thyroid carcinoma remains to be determined.

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ABBREVIATIONS

AA	Amino Acid
ADAMs	adamalysins; A Disintegrase and Metalloproteinase
AG	Antigen
AB	Antibody
APC	Adenomatosis Polyposis Coli
bp	base pair
BSA	Bovine Serum Albumine
BRCA1	breast cancer 1
cAMP	cyclo-Adenosine monophosphate
CD	Cluster of Differentiation
CAMs	Cell Adhesion Molecules
DD	Death Domain
DNA	Deoxyribonucleic Acid
DAB	3, 3-Diaminobenzidine
DED	Death Effector Domain
DEPC	Diethylpyrocarbonate
DISC	Death Inducing Signaling Complex
DMSM	Dulbecco's Minimum Essential Media
DNA	Deoxynucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECM	Extra Cellular Matrix
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	Epidermal Growth Factor
ESI-MS/MS	electrospray ionisation-tandem mass spectrometry
FADD	Fas Associated Death Domain
Fas	Fibroblast associated
FasL	Fas Ligand
FBS	Fetal bovine serum
FTC	Follicular thyroid carcinoma
HBSS	Hank's Balanced Salts Solution
HIV	Human Immundeficiency Virus
h	hour
ICE	Interleukin Converting Enzyme
IGF	Insulin-Like Growth Factor
IgG	Immunglobulin G
kDa	kilo Dalton
MTT	3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MALDI-ToF	matrix-assisted laser desorption ionisation time of flight

multinod.	Multi nodular
min	minute
ml	Milliliter
MMPs	Matrix metalloproteinases
µg	microgram
µl	microliter
MMP	Matrix- Metalloproteinase
NFκB	Nuclear Transcription Factor- kappa B
ng	nanogram
NGF	Nerve Growth Factor
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-Buffered-Saline
PCD	Programmed Cell Death
PDGF	platelet-derived growth factor
PTC	Papillary thyroid carcinoma
PTEN	phosphatase and tensin homolog deleted on chromosome 10
Phe	Phenylalanine
RA	Retinoic Acid
RB1	retinoblastoma 1
resp.	respectively
RGD	Integrin Recognition Motif
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
Tag	Termophilus Aquarius
TBE	TRIS-Boric acid-EDTA
TGFβ1	Transforming Growth Factor β1
TGI	Thyroid Growth Immunoglobulin
TM4SF	Transmembrane-4 Superfamily
TNF	Tumor Necrosis Factor
TNFSF	Tumor Necrosis Factor Superfamily
TNFR	Tumor Necrosis Factor Receptor
TNFRSF	Tumor Necrosis Factor Receptor Superfamily
TSH	Thyroid Stimulating Hormon
TSI	Thyroidea Stimulating Immunoglobulin
UICC	Union Internationale Contre le Cancer
UTC	Undifferentiated thyroid carcinoma
VEGF	Vascular Endothelial Growth Factor

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1 INTRODUCTION

1.1 Molecular fundamentals of tumor growth and metastasis

1.1.1 Tumor metastasis suppressor gene

Tumor metastasis suppressor genes are functionally inactivated as tumor cells acquire metastatic ability and may have significant prognostic value as markers of tumor metastatic potential. Mutation or deletion of a tumor metastasis suppressor gene will increase the probability for the formation of tumor metastasis and turn a tumor suppressor gene into an oncogene. An oncogene is a modified gene that increases the malignancy of a tumor cell. Usually involved in early stages of cancer development, some oncogenes increase the chance of a normal cell to develop into a tumor cell, possibly resulting in cancer.
The Retinoblastoma protein (pRb) was the first tumor suppressor to be discovered in human retinoblastoma disease. Inactivation of pRb by a mutation in both alleles of the coding Rb1 gene can result retinoblastoma. Another important tumor suppressor is the p53 (tumor protein 53) gene product. P53 is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor. Other successfully identified tumor suppressors, e.g., APC, PTEN, NM-23, BRCA1, are involved in cell adhesion, prevent tumor cells from dispersing, block loss of contact inhibition and inhibit metastasis.

1.1.2 Metastasis

Metastasis is the major cause of death in cancer patients. The spread of cancer cells from a primary tumor to secondary sites within the body is complex multi step processes which involve motility, invasion, adhesion, proliferation and angiogenesis. These processes are displayed in Figure 1.

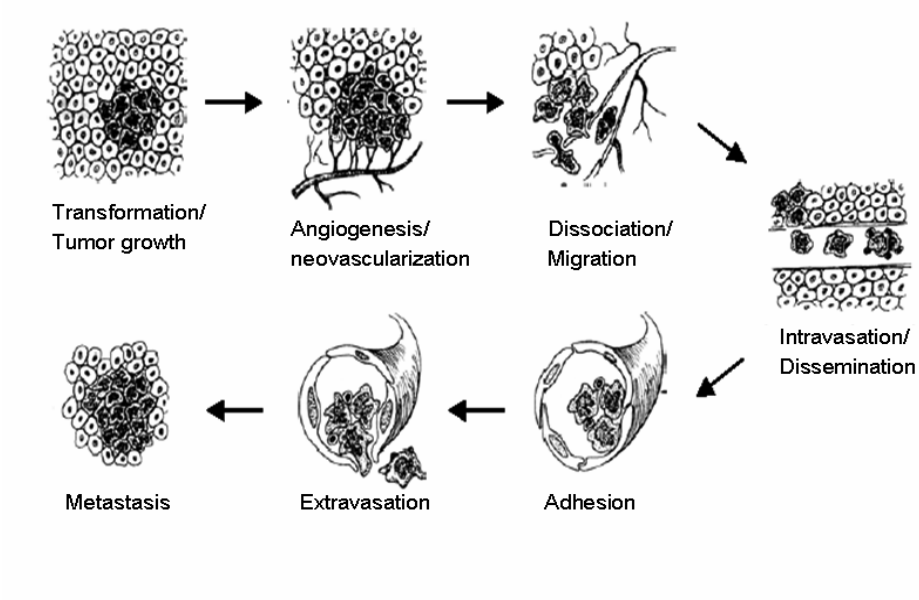


Figure 1: Schematic illustration of the metastasis cascade: The oncogenic transformation and growth of primary tumor is followed by successive steps leading to metastases, including angiogenesis and neovascularization, the dislodgement of tumor cells, migration/intravasation as well as cell adhesion and extravasation of tumor cells. This will eventually lead to the formation of a secondary tumor sites.

Once metastatic cells are attached to the basement membrane, they break through with the help of different enzymes (e.g. type IV collagenase, elastases). Cancer cells then move through the blood stream enabling them to spread to other parts of the body. Malignant cells break away from the primary tumor and attach to and degrade proteins that make up the surrounding extracellular matrix (ECM) which separates the tumor from adjoining tissue. By degrading these proteins, cancer cells are able to breach the ECM and escape. One of the critical events required for cancer metastasis is the creation of a new network of blood vessels. This process of forming new blood vessels is called neoangiogenesis. The formation of blood vessels is required not only to support the growth of primary tumors, but also its pervasion to other organs. The cells dissociate from the primary tumor and migrate in the blood or lymph vessels (intravasation). The dissociation process will be realized by the down-regulation in the expression of some cell membrane molecules expression, e.g. E-Cadherin, cytoskeleton proteins and integrins. These molecules serve as mediators of signal transduction between the intra- and extracellular compartments.

For the entrance into the blood vessels the tumor cells must not only be mobile but must also be able to cross the barrier of the basal membrane. This process is facilitated by the formation and/ or activation of proteases, e.g. cathepsins, proteinases and matrix metalloproteinases [79]. After intravasation into the blood and lymph stream, tumor cells can spread to different body locations (dissemination). However, only an estimated 0,1% of these tumor cells will actually survive [125]. Micrometastases generate frequently from a single tumor cell clones in one of more organs and although posing a potential thread, may not always result in metastases.

1.1.3 Cell-adhesion molecules

Cell adhesion molecules (CAMs) are proteins located on the cell surface facilitating cell-cell interaction and binding to the extracellular matrix (ECM) in a process called cell adhesion. CAMs are typically transmembrane receptors and are composed of three domains: an intracellular domain that interacts with the cytoskeleton, a transmembrane domain and an extracellular domain that interacts either with CAMs of the same kind (homophilic binding) or with different CAMs or the extracellular matrix (heterophilic binding).

Integrins are a family of ubiquitously expressed transmembrane glycoproteins that exist as α/ß heterodimers found at cell adhesion sites known as focal adhesion contacts. They can bind immunoglobulin superfamily (IgSF) CAMs or extracellular matrix components. Eighteen different alpha subunits are known that can link in many different combinations with the 8 different beta subunits. However, not all possible α/ß heterodimer combinations have been described. The integrin extracellular domains function as cell surface receptors for ECM molecules, whereas their intracellular domains connect directly or indirectly via linker proteins to the actin cytoskeleton [50, 2]. Acting in tandem with other signalling pathways, integrins are pivotal in controlling cell attachment, cell migration, cell cycle progression and apoptosis.

Members of cadherin family are calcium-dependent homophilic CAMs, E-cadherins (epithelial), P-cadherins (placental) and N-cadherins (neural) are the most important members of this family. CAMs play a major role in morphogenesis and organogenesis in vertebrates because they are the key factors in mediating cell–cell interaction and cell–matrix interaction. CAMs not only can elicit its specific signal but also can interact with growth factor receptor and other membrane protein.

TM4SF belongs to such transmembrane cell adhesion proteins family. The extracellular domain of a cell adhesion protein can bind to other molecules that might be either on the surface of an adjacent cell (cell-to-cell adhesion) or part of the extracellular matrix (cell-to-ECM adhesion). Cell adhesion proteins are important for the normal functioning of living organisms. Cell adhesion proteins hold together the components of solid tissues. They are also important for the function of migratory cells. Some cancers involve mutations in genes for adhesion proteins that result in abnormal cell-to-cell interactions and tumor growth.

1.1.4 Proteinases

Malignant cells produce a broad spectrum of matrix-degrading enzymes; predominant among these proteinases are enzymes of the plasminogen activator and matrix metalloproteinase family. The extracellular matrix is both the structural scaffolding that establishes and maintains tissue architecture, and an information-processing medium that controls cell position, identity, proliferation and fate. Thus, ECM remodelling is a critical aspect of normal tissue development and repair. Regulated turnover of ECM structures is achieved through

the interplay of degradative enzymes and their natural inhibitors, including the matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs). Loss of control of the MMP-TIMP balance is a factor in the pathological tissue destruction seen in human diseases, including cancer, arthritis and cardiovascular disease. Matrix metalloproteinases (MMPs) comprise a class of enzymes that degrade the extracellular membrane components such as collagenase, gelatinase, and stromelysins. Recently developed synthetic metalloproteinase inhibitors (MPIs) have shown some efficacy in cancer trials, but these inhibitors have exposed the existence of another family of metalloproteinases, the adamalysins (ADAMs) that function in cell adhesion, signalling and the processing of cytokines and their receptors. These enzymes are believed to have an essential role in the metastatic process, particularly in those steps which require tumor cells to traverse the basement membrane and the stroma around blood vessels. MMPs and TIMPs are secreted by both tumor cells and stromal cells [126, 67, 16]. It is currently believed that an imbalance between active MMPs and TIMPs causes degradation of the basement membrane and allows angiogenesis, tumor growth, and invasion to occur. Therefore, synthetic MMP inhibitors are being developed for their potential antimetastatic and antiangiogenic properties [19].

1.1.5 Angiogenesis

Tumor angiogenesis involves the proliferation of endothelial cells and the creation of a network of blood vessels which penetrate tumor tissues, supplying nutrients and oxygen and removing waste products. Tumor angiogenesis can be initiated by tumor cells releasing molecules that encourage growth of new blood vessels within the cancer and surrounding normal tissues. Angiogenesis is essential for tumor growth and progression. Accumulating evidences indicates that progressive tumor growth is dependent on angiogenesis. Leaky blood vessels often found in tumor tissues cause an increase of interstitial pressure that limit drug diffusion within the tumor and favour tumor cell dissemination in the blood stream [34, 12]. Eventually, this will induce tumor spreading and the appearance of tumor metastases.
Tumor angiogenesis is thought to result from the secretion of "angiogenesis factors" by tumor cells; these include growth factors, cytokines, and also a number of small molecules. Vascular Endothelial Growth Factor (VEGF) is well understood to be a major contributor to increasing the number of capillaries in a given network. *In vitro* studies clearly demonstrate that VEGF is a potent stimulator of angiogenesis because in the presence of this growth factor plated endothelial cells will proliferate and migrate, eventually forming tube structures resembling capillaries. VEGF causes a massive signaling cascade in endothelial cells. Binding to VEGF receptor-2 (VEGFR-2) starts a tyrosine kinase signaling cascade that stimulates eNOS expression and begins producing nitric oxide (NO). Mechanically, VEGF is

upregulated with muscle contractions as a result of increased blood flow to affected areas. The increased flow also causes a large increase in the mRNA production of VEGF receptors 1 and 2. The increase in receptor production means that muscle contractions could cause upregulation of the signaling cascade relating to angiogenesis. As part of the angiogenic signaling cascade, NO is widely considered to be a major contributor to the angiogenic response because inhibition of NO significantly reduces the effects of angiogenic growth factors. However, inhibition of NO during exercise does not inhibit angiogenesis indicating that there are other factors involved in the angiogenic response.

Angiogenesis can be viewed as the result of a dynamic balance of tightly regulated oncogenes and suppressor genes, stimulatory and inhibitory peptides, proteases and endogenous inhibitors, and microenvironmental factors such as the level of oxygen or copper ion. The other principal growth factors driving angiogenesis are bFGF, and hepatocyte growth factor [9, 93]. Other positive regulators are angiopoietin-1, angiotropin, angiogenin, epidermal growth factor (EGF), granulocyte colony-stimulating factor (GCSF), interleukin (IL)-1, IL-6 and IL-8, platelet-derived growth factor (PDGF), and tumor necrosis factor-alfa (TNF-alpha) [20, 111].

1.1.6 Cytoskeletal proteins

The major filamentous proteins of the cytoskeleton are represented by tubulins, actin microfilaments, and intermediate filament (IF) proteins. Each type is build of specific proteins and is distinguished by its diameter and function. Actin microfilaments and microtubules function to maintain cell shape and enable intracellular transport, while IFs are speculated function as signal and/or energy transducers between the extracellular matrix and cell nucleus. Furthermore, there is evidence that the extracellular matrix can regulate gene expression via IFs, thus modulating cellular functions. Intermediate filament proteins are specifically regulated during embryonic development and cellular differentiation. Vimentin is typical for mesenchymal cells while keratin characterizes epithelial cells. Microtubules are tubes made up of spiraling, two-part subunits. It is made of tubulin. It aids in mitosis/meiosis and movement of organelles, cilia and flagella. Changes in the cytoskeleton are observed in cancer cells. Cancer cells often show increased movement. In fact, metastatic spread of cancer is dependent on tumor cells that invade neighboring tissues.

The relationship between cell attachment to the substratum and cytoskeletal organization can affect cell survival and transformation. Pascal Seve et al. reported a high expression of class III h-tubulin in tumor cells is associated with resistance to chemotherapy and a poor prognosis in patients with non–small cell lung cancer receiving vinorelbine-based chemotherapy [99, 115]. The state of tubulin polymerization associates with tumor metastasis and increased depolymerized form of tubulin could promote metastasis. Changes

in the expression of genes for the cytoskeleton organization and biogenesis mediate adaptation to increased motility and invasion of the metastatic tumor cell.

1.2 Introduction to TM4SF members

Members of the tetraspanin superfamily proteins expressed in a wide range of human cells. The TM4SF includes at least 16 core members and a number of additional proteins with sequence similarities. Nearly all mammalian cells contain large numbers of one or more TM4SF proteins. One of the main functions seems to be the regulation of interactions between cells by stabilization of multimeric protein complexes on the cell-surface. TM4SF have effects on cell proliferation, motility, and adhesion many type of cells. They have been implicated in a multitude of biological processes, but their distinct function remains unclear. However, it has been reported that several TM4SF members, such as CD9, CD63, CD81, CD82 and CD151 might be involved in cell signalling. Further data suggest a function in cell proliferation and tumour cell metastasis.

Table 1: Major Tetraspanin family members and associated functions

Approved Symbol	Alias	Gene bank ID	Location	Function
CD9	DRAP-27, MRP-1	M38690	12p13	modulate cell adhesion and migration
CD37	gp52-40	X14046	19p13-q13.4	T-cell-B-cell interactions
CD53	MOX44	M37033	1p13	regulate cell growth
CD63	NGA, ME491, gp55, LIMP, MLA1, PTLG, P40, LAMP-3	M58485	12q12-q13	regulate cell development, activation, growth and motility
CD81	TAPA-1	M33680	11p	regulate cell development, activation, growth and motility
CD82	KAI1, ST6, SAR2, R2, IA4, GR15, C 33, 4F9	U20770	11p11.2	metastasis suppressor gene
CD151	SFA-1 PETA-3	U14650	11p15.5	enhances cell motility, invasion and metastasis of cancer cells
TM4SF2	DXS1692E, TALLA-1, Hs.82749, A15, CCG-B7, MXS1	D10653	Xq11	unknown
TM4SF3	CO-029	M35252	12	unknown
TM4SF6	TSPAN-6, T245	AF043906	Xq22	unknown
TM4SF7	NAG-2	AF022813	11p15.1-q15.5	unknown

1.2.1 Tetraspanin gene structure

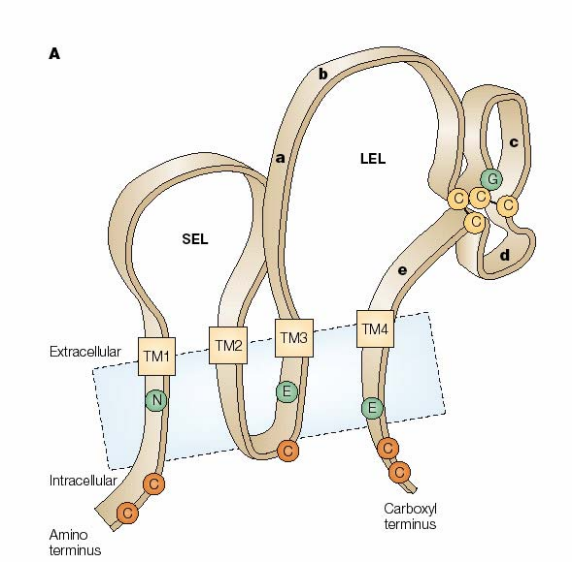


Figure 2: Schematic structure of tetraspanins according to Shoshana Levy et al. The four transmembrane (TM) domains contain conserved polar residues (green circles) and flank the small and large extracellular loops (SEL and LEL, respectively). The LEL is composed of a core formed by helices a, b and e, and this core structure is conserved among the tetraspanins. Helices c and d comprise the variable portion of the LEL, and they are flanked by the CCG motif and further conserved cysteine residues (yellow circles). This region is folded as a result of disulphide bridges (black lines) to form a mushroom-like structure. Potential palmitoylation sites on the intracellular conserved cysteine residues are indicated in orange circles.

As type III (N- and C- terminus intracellular) integral membrane protein, CD82 and CD63 have two divergent extracellular loop domains, the larger of which contains several conserved amino acid motifs, and two short cytoplasmic domains at the NH2 and COOH termini [24]. The human CD82 gene (KAI1: kangai 1) located on human chromosome 11p11.2 was originally identified in human prostate carcinoma based on its function as a metastasis suppressor gene.

Dong et al. reported that the CD82 gene encoding 10 exons is contained within 80 kb of DNA. Among several other members of the 'transmembrane 4 superfamily,' including CD82, the locations of the splice sites relative to the structural domains of the encoded protein are conserved [23]. CD82 encodes 267 amino acids with four putative transmembrane domains (>23 aa) separating two unequal extracellular loops (27, 119 aa). The two short N´- (9 aa) and C´-terminal (15 aa) ends are located in the cytoplasm.

Sequencing of the CD82 cDNA clone revealed complete identity to human lymphocyte surface antigen named R2, IA4, 4F9 or C33. The gene and protein structure of the CD82 have been reviewed recently [24, 17]. The large extracellular hydrophilic domain is stabilized by disulfid-bridge and is heavily glycosylated attributed to N-glycosylation which has been shown to depend on the cell type and state of cell activation. Thus, glycosylation results in CD82 reaching a molecular weight of up to 80 kDa.

The human melanoma-associated antigen CD63 (alternative names: NGA, ME491, gp55, LIMP, MLA1, PTLG, P40, LAMP-3) is expressed strongly during early stages of progression of the tumor. CD63 was firstly cloned and reported by Hotta et al. [78]. The sequence of the cDNA indicates that the antigen has 237 amino acids (molecular weight 25,475) with 4 transmembrane regions and 3 putative N-glycosylation sites and shows a cell-type specific glycosylation. The gene was mapped to 12p12-q13 by somatic cell hybrid analysis and to 12q12-q14 by in situ hybridization [78].

1.2.2 Function

CD82 appears to have a central role in controlling metastasis as a 'molecular facilitator'. In epithelial cells, CD82 is implicated in diverse biological processes such as cell adhesion, migration, apoptosis and morphogenesis. Complex mechanisms underlie CD82 loss of functions and include altered transcriptional regulation, splice variant production and post-translational protein modifications.

Mashimo et al. [71] reported that the tumor suppressor gene p53 can directly activate the CD82 gene by interacting with a 5-prime upstream region. The data from this study indicated a direct relationship between p53 and CD82 gene activation and suggested that the loss of p53 function commonly observed in many types of cancer can leads to a downregulation of the CD82 gene, thus, propagating of metastatic spreading of tumor cells.

Baek et al. demonstrated that interleukin-1ß causes nuclear export of a specific nuclear receptor corepressor (NCOR corepressor) complex which results in de-repression of a specific subset of nuclear factor kappa-B-regulated genes, among them being CD82. Nuclear export of the NCOR/TAB2/ HDAC3 complex by IL1ß is linked to selective recruitment of a TIP60 (Tat-interactive protein 60kDa) coactivator complex. TIP60, one of the best characterised MYST proteins, is a human homologue with relevance to human pathology. CD82 is also directly activated by a ternary complex, dependent on the acetyltransferase activity of TIP60 the presenilin-dependent C-terminal cleavage product of the beta amyloid precursor protein, and FE65 acting as a ternary complex directly activate the CD82 gene which identifies a specific *in vivo* gene target of an APP-dependent transcription complex in the brain [7]. Kim et al. reported that the downregulation of the metastasis suppressor gene CD82 in prostate cancer cells involves the inhibitory actions of beta-catenin, along with a reptin chromatin remodeling complex. This inhibitory function of beta-catenin-reptin requires both increased beta-catenin expression and recruitment of histone deacetylase activity. The coordinated actions of beta-catenin-reptin components that mediate the repressive state serve to antagonize a TIP60 coactivator complex that is required for activation; the balance of these opposing complexes controls the expression of CD82 and metastatic potential. The molecular mechanisms underlying the antagonistic regulation of ß-catenin-reptin and the

TIP60 coactivator complexes for the CD82 metastasis suppressor gene are likely to be prototypic of a selective downregulation strategy for many genes, including a subset of NF-kappa-B target genes [61].

In gastric cancer patients that loss of CD82 expression could indicate metastasis potential and the poor clinical outcome [117], and a significantly reduced mRNA expression of CD82 was found in breast cancer brain metastases [109]. CD82 suppresses integrin-induced invasion by regulating signaling to c-Met and Src kinases and suggested that loss of CD82 may promote metastasis by removing a negative regulator of c-Met and Src signalling [108], and the activity of CD82 promoter is dependent on p53, junB and AP2 [70]. In bladder cancer, the expression of the CD82 associated with invasiveness of bladder carcinoma cells metastasis suppressor [128, 56]. CD82 has also been linked to carcinogenesis and metastasis in tumors of the bone and soft tissues and neuroblastoma [5, 6].

Thus, CD82 has established itself as a clinically significant factor associated with tumor cell migration in different human malignant neoplasms.

Table 2: association of CD82 expression with human cancers

Cancer type	Study number	downregulation	upregulation	Prognosis(+)
Bladder	3	3	0	1
Bone and soft tissues	1	1	0	-
Breast	3	3	0	1
Cervix	3	3	0	1
Endometrial	1	1	0	1
Gastrointestinal	16	12	4	7
Head and neck	1	1	0	-
Leukemia	1	0	1	-
Liver	1	1	0	1
Lung (NSCLC)	8	7	1	5
Neuroblastoma	2	2	0	1
Oral (OSCCs)	3	3	0	2
Ovary	3	3	0	2
Pancreas	5	4	1	3
Papilla of Vater	2	-	-	-
Prostate	8	6	2	3
Skin	1	1	0	-

Prognosis (+): positive prognosis

Only two studies on CD82 expression in human papillary thyroid carcinoma (PTC) have been conducted so far. Ito et al. presented that CD82 down-regulation is positively related to the progression of PTC, occurrence of lymph node metastases and anaplastic transformation of PTC. In a recent clinical investigation of 19 microcarcinoma patients with clinically apparent metastasis, 14 patients with not assessable metastasis, and 22 patients without metastasis the same group showed that CD82 expression was significantly reduced in metastasis patient tissues [54, 53].

CD63, also known as granulophysin and melanoma antigen ME491, is expressed in several normal tissues and cells (dysplastic nevi, radial growth phase primary melanomas, hematopoietic cells, tissue macrophages) as well as in most cultured melanoma cell lines [74]. Interestingly, CD63 is strongly expressed in early stages of melanoma but its expression is weaker or absent in late malignant stages suggesting some role of CD63 in malignant progression of melanoma [63]. Transfection of CD63 into a CD63-negative melanoma cell line reduces metastasis *in vivo* and this effect is associated with the suppression of tumorigenicity and cell motility [87, 88]. Although conclusive proof is currently lacking, it appeared that the CD63 down-regulation during malignant transformation of melanoma cells CD63 expression may inhibit metastasis of human melanomas. Nishibori et al. demonstrated a deficiency of CD63 protein in Hermansky-Pudlak Syndrome (HPS) [81]. Clarification as to whether deficiency in CD63 is the primary defect in HPS will await molecular characterization of the CD63 gene in HPS patients. A primary defect in the CD63 gene is unlikely because the HPS gene maps to chromosome 10, not to chromosome 12 as for the CD63 gene [35]. Erdmann et al. found a positive correlation between IgE reactivity and the number of CD63 positive basophils for all food allergens. They conclude that quantification of basophil activation by CD63 expression is a valuable new in-vitro method for diagnosis of immediate type food sensitization [29]. Expression of CD63 was also detected distal to the epithelium of the stomach, duodenum, small intestine, and colon [83]. In human bone marrow mast cells high amounts of CD63 are present with most CD63 being located intracellularly. Patients with mastocytosis also display higher CD63 levels [31].

1.3 Epidemiology of malignant thyroid tumor in Germany

Thyroid carcinoma is the 12th most common malignant disease in Germany and occurs with an incidence of 3/100,000 annually. Three different types of thyroid carcinoma can derive from thyrocytes and are well classified. Differentiated tumors (papillary and follicular) are amendable to treatment and usually curable. Papillary (PTC) and follicular thyroid carcinoma (FTC) account for 80-90% of all thyroid cancers. PTC is the most common of thyroid malignancies occuring at all age groups with a particular prevalence in women under 45 years of age. The prognosis is usually optimistic with long-term survival rates of more than 90%. However, some PTCs behave more aggressively with recurrences and/or distant metastases and have a poor prognosis. The second-most frequent FTC tends to spread into lungs and bones. Poorly differentiated aggressive FTC is rare, metastasize early and have a poor prognosis. Rare undifferentiated (anaplastic) thyroid carcinoma (UTC) is the most aggressive thyroid malignancy with an extremely poor 5 years survival rate of fewer than 5%. Patients with a history of radiation administered in infancy and childhood for benign conditions of the head and neck, such as enlarged thymus, acne, or tonsillar or adenoidal

enlargement, have an increased risk of thyroid cancer as well as other abnormalities of the thyroid gland. In this group of patients, malignancies of the thyroid gland first appear beginning as early as 5 years following radiation and may appear 20 or more years later. Radiation exposure as a consequence of nuclear fallout as in the case of the Chernobyl disaster has also been associated with a high risk of thyroid cancer, especially PTC in children [86, 18]. Other risk factors for the development of thyroid cancer include a history of goiter, family history of thyroid disease, female gender, and Asian race [52].

1.4 Current molecular factors in thyroid carcinoma

The prognosis for differentiated carcinoma is better for patients younger than 40 years without extracapsular extension or vascular invasion [40, 90, 73, 110, 72]. Age appears to be the single most important prognostic factor [73]. The prognostic significance of lymph node status is controversial. One retrospective surgical series of 931 previously untreated patients with differentiated thyroid cancer found that female gender, multifocality, and regional node involvement are favorable prognostic factors [100]. Adverse factors included age older than 45 years, follicular histology, primary tumor diameter bigger than 4cm (T3), extrathyroid extension (T4), and distant metastases [100, 4]. Other studies have shown that regional lymph node involvement had no effect [21, 119] or even an adverse effect on survival [100, 4, 98]. Diffuse intense immunostaining for vascular endothelial growth factor (VEGF) in patients with PTC has been associated with a high rate of local recurrence and distant metastases [66]. An elevated serum thyroglobulin level correlates strongly with recurrent tumor when found in patients with differentiated thyroid cancer (PTC or FTC) during postoperative evaluations [89]. Serum thyroglobulin levels are most sensitive when patients are hypothyroid and have elevated serum thyroid-stimulating hormone levels [27]. Expression of the tumor suppressor gene p53 has also been associated with an adverse prognosis for patients with thyroid cancer [38].

Investigations by Hoang-Vu et al. showed thyroglobulin (Tg) and thyroid peroxidase (TPO) mRNA were strongly expressed in normal tissues and completely lost in UTC tissues [47]. Brabant et al. reported that E-cadherin served as a differentiation marker in thyroid malignancies [15]. E-cadherin expression seems to be associated with the dedifferentiation, progression, and metastatic spread of thyroid carcinomas and may be a useful marker for the prognosis of these tumors [61, 91]. Investigations of Hoang-Vu et al. concerning the telomerase RNA component (hTR), telomerase associated protein (TP1) and the telomerase catalytic subunit (hTERT) in human thyroid indicated that the comparisions of TP1 and hTR, hTERT and telomerase activity may be helpful in identifying invasive tumors and may be additional markers for classification of benign goiter and malignant thyroid carcinoma [46]. Recently, Boltze et al. reported the maspin possibly functions as a clinically relevant inhibitor of tumor progression, preventing local invasiveness and further systemic progression of

papillary thyroid carcinomas [13]. Kehlen et al. showed that the APN/CD13-associated the down-regulation of NDRG-1, ME491/CD63, and DPIV/CD26 in thyroid carcinoma cells is an important step in tumor progression to more malignant phenotypes, and APN/CD13 was indentified as an important mediator in a multi molecular process regulating malignant thyroid cell migration [60]. Aldred et al. presented functional data indicating that the downregulation of PPARgamma is a key event in multiple types of thyroid neoplasia and possible target for therapeutic intervention [3]. Siironen et al. investigated the expression COX-2, MMP-2, VEGF-C, Bcl-2, Ki-67, and p21 in thyroid carcinoma tissues of 601 consecutive patients. None of these markers showed superiority over TNM classification in selecting patients likely to progress to aggressive disease [106]. Fernandez-Real et al. reported that the high ICAM-1 protein production in distant metastastic thyroid carcinoma [32]. Figge et al. reported that papillary carcinomas displayed significant higher CD44 antigen than in follicular epithelium from goiter tissues [33]. Kebebew et al. reported that ECM1 and TMPRSS4 could be the excellent diagnostic markers of malignant thyroid nodules and may be used to improve the diagnostic accuracy of FNA biopsy [59].

1.5 Postoperative histopathological classification (pTNM)

Thyroid tumors were staged according to the Tumor-node-metastasis (TNM) staging classification (UICC-AJCC 1997).

I - Primary tumor (T or pT = histopathologic)

- pT0 : no evidence of primary tumor
- pT1 : tumor 1 cm or less in greater dimension, intra-thyroid
- pT2 : tumor more than 1cm but no more 4 cm in greater dimension, intra-thyroid
- pT3 : tumor more than 4 cm in greater dimension, intra-thyroid
- pT4: tumor of any size extending beyond the thyroid capsule

II - Regional lymph nodes (N or pN)

- pN0 : no regional lymph node
- pN1 : regional lymph node metastasis
  - N1a : ipsilateral
  - N1b : bilateral, midline or controlateral cervical or mediastinal lymph nodes

III - Metastasis (M or pM)

- pM0 : no metastasis
- pM1: distant metastasis

1.6 Benign thyroid goiter

The term nontoxic goiter refers to an enlargement of the thyroid which is not associated with (a) the overproduction of thyroid hormone or (b) malignancy. The thyroid can become very large so that it can easily be seen as a mass in the neck. There are a number of factors which may cause the thyroid to become enlarged. A diet deficient in iodine can cause a goiter but this is rarely the cause because of the readily available iodine in our diets. A more common cause of goiter in America is an increase in thyroid stimulating hormone (TSH) in response to a defect in normal hormone synthesis within the thyroid gland. The thyroid stimulating hormone comes from the pituitary and causes the thyroid to enlarge. This enlargement usually takes many years to become manifest. Goiter is often merely a symptom of a more serious thyroid condition, such as:

- Hyperthyroidism, an overactive thyroid gland;
- Hypothyroidism, an underactive thyroid gland;
- Thyrotoxicosis, an excessive amount of thyroid hormone in the bloodstream;
- Graves' disease (also known as toxic diffuse goiter or exophthalmic goiter).

2 OBJECTIVES

The purpose of this study was to determine the role of two TM4SF members, CD82 and CD63, for cellular differentiation, adhesion and tumor metastasis in thyroid carcinoma. This project was divided into three parts as mentioned below:

2.1 Clinical significance of CD82/ CD63 in human thyroid:

Currently, there are only two investigations on CD82 in human thyroid carcinoma reported by Japanese research groups [54, 53]. Kehlen et al. reported that the overexpression of APN/CD13 is associated with down-regulation ME491/CD63 gene expression in FTC-133 cells [60]. In the present retrospective study on staged human thyroid carcinoma tissues the aim was to investigate systematically whether (a) CD82 and CD63 may serve as novel molecular diagnostic/ prognostic markers in human thyroid carcinoma and whether (b) CD82 and CD63 could suppress the invasive and metastatic capability of thyroid carcinoma. By using semi-quantitative RT-PCR and immunohistochemistry techniques, we studied the transcript and protein expression levels of 87 patient surgical specimens. To find correlations, we compared the expression levels for CD82 and CD63 with different clinical pathological parameters.

2.2 *In vitro* investigation of TM4SF's biological function

CD82-overexpressing FTC-133 models were etablished by a plasmid construct-transfection approach to further characterize the functions of this TMSF4 member in the human thyroid and the interaction between TM4SF with other membrane and cytoskeleton proteins. The coding sequence of CD82 cDNA was subcloned into the eukaryotic expression plasmid pcDNA3.1 and transfected in human follicular thyroid carcinoma cells (FTC-133). Once the cells stable overexpressed CD82, the viability and motility of wild type and transfected cells were evaluated by MTT and modified Boyden Chamber assays. Immunofluorescence staining was used for the investigation of the cellular protein localization.

2.3 Proteomic investigations of FTC-133 cells

To define alterations through CD82 overexpression in gene expression within a significant fraction of proteome investigation of FTC-133 cells, two-dimensional electrophoresis of isolated total proteins from wild type and transfected cells were performed. The gels were stained with silver nitrate and protein spots in each gel were compared for differences in spot presence/absence and spot intensity. Finally, mass spectrometry (MALDI-TOF/Q-TOF) was used for protein identification. As a long-term goal of this investigation, we might detect

known or unknown proteins which associate with CD82 and affect tumor cell differentiation, apoptosis, adhesion and tumor metastasis.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and biochemical reagents

Table 3: List of chemicals and biochemical reagents used in this study

Chemicals and biochemical reagents	Source
acidic acid	VWR, Darmstadt
Agarose	Roche Molecular Biochemicals, Mannheim
Ammoniumpersulfat (APS)	Pharmacia Biotech, Freiburg
AmpliTaq polymerase-Gold	Roche, USA
Bromophenol Blue	GE Healthcare, life science, Sweden
Bovines Serum albumin	Sigma-Aldrich, Steinheim
Bromphenolblau	SERVA, Heidelberg
Chloroform	MERCK, Darmstadt
CHAPS	GE Healthcare, life science, Sweden
Cleaning solution	GE Healthcare, life science, Sweden
Dithiothreitol	Carl Roth, Karlsruhe
EDTA	Merck, Darmstadt
Ethanol	Merck, Darmstadt
Ethidiumbromid	SERVA, Heidelberg
Glycine	SERVA, Heidelberg
G418- Sulfat (Genitincin)	Invitrogen, UK
HCl	VWR, Darmstadt
Hydrogen peroxide solution, 30%	MERCK, Darmstadt
Isopropyl alcohol	MERCK, Darmstadt
Iodoacetamide	Sigma-Aldrich, Steinheim
Lipofectamin 2000	Invitrogen, UK
Methanol	VWR, Darmstadt
Mayer's Hemalum solution	MERCK, Darmstadt
NaHCO3	MERCK, Darmstadt
Natrium carbonate	Carl Roth, Karlsruhe
Natrium thiosulfate	Carl Roth, Karlsruhe
PBS	AppliChem GmbH, Darmstadt
PlusOne™ Glycine	GE Healthcare, life science, Sweden
PlusOne™ TEMED	GE Healthcare, life science, Sweden
PlusOne™ SDS	GE Healthcare, life science, Sweden
peQ Universal Agarose	PeQLab Biotechology, Erlangen
Pharmalyte	GE Healthcare, life science, Sweden
Protease inhibitor cocktail tablets	Roche Diagnostics GmbH, Mannheim
rTaq polymerase	GE Healthcare, life science, UK
Retinoic acid	Sigma-Aldrich, Steinheim
Random primer	Invitrogen, UK
RNAse free Water	Qiagen, Hilden
RNAse out	Invitrogen, UK
X-ray film (Hyperfilm)	GE Healthcare, life science, UK
Silver nitrate solution	Carl Roth, Karlsruhe

Sodium Dodecylsulfate	GE Healthcare, life science, Sweden
SDS (Natriumdodecylsulfat)	Sigma-Aldrich, Steinheim
Triton X-100	Sigma-Aldrich, Steinheim
Tris	GE Healthcare, life science, Sweden
TEMED	Biorad, München
Thiourea	Fluka, Steinheim
TWEEN 20	SERVA, Heidelberg
TRIZOL reagent	Invitrogen, Carlsbad, USA
Trypsin/ EDTA	Gibco, Berlin
Urea	GE Healthcare, life science, Sweden
2- methyl-1-propanol	Carl Roth, Karlsruhe
3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide	Sigma-Aldrich, Steinheim

3.1.2 Buffers

Table 4: List of buffers and their composition used in this study

Standard buffers	Components
PBS	137mM NaCl; 2,7mM KC; 4,3mM Na2HPO4x7H2O, 1,4 mM KH2PO4, pH 7,4
10xTBE	890mM Tris-Base, 890mM Boric acid, 20mM EDTA, pH 8,0
TAE	0,4M Tris Base; 0,2M cold acidic acid; 0,1M EDTA, pH 8,4
Protein extraction buffer (western blot)	5M NaCl, 1M TRIS/ HCL pH 7,5, 0,5M EDTA pH 8,0, 0,5M EGTA pH 7,5, Triton-100, Protease inhibitor cocktail tablets (1 tab/10ml lyses buffer)
Sample preparation solution (2D)	8M UREA, 2M Thio UREA, 4% CHAPS, 40mM DTT, 0,5% Pharmalyte IEF 3-10
Sample puffer (western blot)	7M Urea, 1% SDS, 0.35M NaCl, 10mM EDTA, 10mM Tris pH 7.5
Loading buffer	0,5M TRIS/ HCL pH 6,8, Glycerin, 10% SDS, Bromphenolblue, Aqua bidest
TBST buffer	10 mM Tris 0.5M NaCl, pH 7.5, 1ml TWEEN 20/ 1L buffer
TBS buffer	10mM Tris pH 7.5

3.1.3 Instruments

Table 5: List of instruments used in this study

Cell laboratory	
Cell incubator, Herasafe	Heraeus Instruments
Safety cabinet, HS 12	Heraeus Instruments
Cool centrifuge, Hettich POTANTA/RP	Heraeus Instruments
Water bath box, WB14	Memmert GmbH
Light (fluorescence) Microscope, Axiovert 25	Carl Zeiss

2D Gelelektrophorese system	
Ettan™ IPGphor™ II IEF System	GE Healthcare, life science
Ettan™ DALTsix Electrophoresis Unit	GE Healthcare, life science
Mass spectrometry laboratory	
Voyager DE PRO MALDI-TOF workstation	Applied Biosystems
QSTAR® XL Hybrid LC/MS/MS System	Applied Biosystems
RNA laboratory	
Horizontal Gelelektrophorese system	BIO-RAD Laboratories
Homogenizer (MIKRO-DISMEMBRATOR S)	B. Braun Biotech international
3 TRIO – Thermocycler	BIOMETRA
UV-Transilluminator	BIOMETRA
Table micro centrifuge	Denver Instruments
Eppendorf-Thermomixer 5436	FAUST
HIGH-SPEED-centrifuge	Heraeus Instruments
Kodak scann camera, Image station 440 CF	Kodak
Immunohistochemistry laboratory	
PALM MicroLaser systems	P.A.L.M. Microlaser Technologies
Cryotom (HM 560)	MICROM
Western blot laboratory	
Trans-Blot Cell	BIO-RAD Laboratories
Mini-Protean II device	BIO-RAD Laboratories
WK230 LAUDA cooling system	Boehringer Ingelheim
Measurement laboratory	
Photospectrometer UV 1602	Shimanzu
Spectra Rainbow ELISA	TECAN
Precision balance	Sartorius
pH-Meter	InoLab

3.1.4 Primers

Table 6: List of primers

Amplificant	size	Primer squence	Anneling (°C)
CD82	598 bp	sense GCA GTC ACT ATG CTC ATG G antisense TGC TGT AGT CTT CGG AAT G	58°C
CD63	347 bp	sense CCC GAA AAA CAA CCA CAC TGC antisense GAT GAG GAG GCT GAG GAG ACC	56°C
18S	346 bp	sense GTT GGT GGA GCG ATT TGT CTG G antisense AGG GCA GGG ACT TAA TCA ACG C	60°C
ß-actin	608 bp	sense GCT GGA AGT GGA CAG CGA antisense GGC ATC GTG ATG GAC TCC G	56°C
Integrin α3	657 bp	sense AAG CCA AGT CTG AGA CT antisense GTA GTA TTG GTC CCG AGT CT	60°C
VEGFc-165	377 bp	sense CTG ATG AGA TCG AGT ACA TCT TCA AGC antisense AGC AAG GCC CAC AGG GAT TT	60°C
survivin	398 bp	sense AAC AGC CGA GAT GAC CTC C antisense AAC TTC AGG TGG ATG AGG AGA C	60°C

S100A4	288 bp	sense GAA GGC CCT GGA TGT GAT GGT G antisense CAT TTC TTC CTG GGC TGC TTA TC	62°C
TGFß1	246 bp	sense AAG TGG ATC CAC GAG CCC AA antisense GCT GCA CTT GCA GGA GCG CAC	51°C
MT1-MMP	492 bp	sense ACA TTG GAG GAG ACA CCC AC antisense AGG CAG TGT TGA TGG ACG C	62°C

3.1.5 Antibodies

Table 7: List of antibodies used in this study

Antibodies	Source
Anti-β-actin antibody, mouse monoclonal AC15	Sigma, Steinheim, Germany
Anti-CD82 antibody, mouse monoclonal 50F11	BD Pharmingen
Anti-CD63 antibody, mouse monoclonal NKI/C3	Novocastra Laboratories Ltd.
Anti-mouse IgG-HRP, Sc-2005	Santa Cruz Biotechnology

3.1.6 Kits and Standards

Table 8: Kits and RNA/ DNA and protein standards used in this study

Kits	Source
2-D Clean-Up Kit	GE Healthcare, life science, Sweden
2-D Quant Kit	GE Healthcare, life science, Sweden
Sample Grinding Kit	GE Healthcare, life science, Sweden
Pierce Western Blotting substrate Reagents	Perbio science, Germany
LSAB-Kit-plus	Dako, Germany
High-Range Rainbow Molecular Weight Markers	GE Healthcare, life science, Sweden
100 bp DNA Ladder	Invitrogen, UK
1000 bp DNA Ladder	Invitrogen, UK

3.1.7 Human tissue samples

Thyroid tissue specimens from 75 patients with thyroid carcinoma and 12 patients with benign thyroid goiter, 35 males and 52 females, were investigated in the present study. Tissues of all patients had been obtained after surgery performed between 1994 and 2001 at the Department of General, Visceral and Vascular Surgery, Martin Luther University Halle-Wittenberg, Halle/ Saale, Germany. The mean age of patients was 58 years, with a range of 15-89 years. Tumor tissues were staged according to the Tumor-Node-Metastasis (TNM) staging classification (UICC-AJCC 1997). The specimens were cryopreserved in liquid nitrogen after resection.

Table 9: Clinical and pathological parameters of investigated patient materials:

Patient no.	Gender	Age (years)	Histological diagnosis	pTNM	Staging
1.	Female	47	FTC	pT4N0M0	III
2.	Female	60	FTC	pT4N0M0	III
3.	Male	60	FTC	pT3N1M0	III
4.	Male	73	FTC	pT4N0M0	III
5.	Female	58	FTC	pT3N1M1	IV
6.	Female	67	FTC	pT4N0M1	IV
7.	Female	61	FTC	pT2N0M0	II
8.	Female	89	FTC	pT4N1M1	IV
9.	Male	66	FTC	pT3N0M1	IV
10.	Female	74	FTC	pT3N0M0	II
11.	Female	41	FTC	pT4N0M0	I
12.	Male	18	FTC	pT3N0M0	I
13.	Male	70	FTC	pT4N0M0	III
14.	Female	51	FTC	pT1N0M0	I
15.	Male	71	FTC	pT3N1bM0	III
16.	Female	39	FTC	pT4N0M0	III
17.	Female	73	FTC	pT2N1M0	III
18.	Female	59	FTC	pT4N0M0	III
19.	Male	67	FTC	pT4N0M0	III
20.	Female	39	FTC	pT4N0M0	III
21.	Female	63	FTC	pT4N0M0	III
22.	Female	62	FTC	pT4N1M0	III
23.	Female	67	FTC	pT4N1bM1	IV
24.	Male	63	FTC	pT4N0M0	III
25.	Female	24	PTC	pT2N0M0	I
26.	Female	47	PTC	pT1N0M0	I
27.	Female	81	PTC	pT4N1M0	III
28.	Female	53	PTC	pT2N0M0	II
29.	Female	62	PTC	pT1N0M0	I
30.	Female	59	PTC	pT3N1bM0	III
31.	Female	77	PTC	pT4N0M0	III
32.	Male	78	PTC	pT4N1M1	IV
33.	Female	16	PTC	pT2N1M0	I
34.	Female	70	PTC	pT4N0M0	III
35.	Male	38	PTC	pT3N1M0	I
36.	Female	53	PTC	pT2N1M0	III
37.	Male	31	PTC	pT4N1bM0	I
38.	Male	71	PTC	pT3N1M0	III
39.	Female	32	PTC	pT4N1bM0	I
40.	Male	58	PTC	pT4N1aM1	IV
41.	Male	30	PTC	pT4N1bM0	I
42.	Male	42	PTC	pT3N0M0	I
43.	Male	71	PTC	pT4N1bM0	III
44.	Female	68	PTC	pT4N0M0	III
45.	Female	62	PTC	pT4N0M0	III
46.	Female	63	PTC	pT4N1M0	III
47.	Female	35	PTC	pT2N1bM0	I
48.	Female	76	PTC	pT3N1M0	III

49.	Male	38	PTC	pT1N0M0	I
50.	Female	45	PTC	pT4N0M0	III
51.	Female	57	PTC	pT1N0M0	I
52.	Female	79	PTC	pT4N1M0	III
53.	Male	67	PTC	pT2N1M0	III
54.	Female	25	PTC	pT4N1bM0	I
55.	Female	56	PTC	pT2N0M0	II
56.	Female	30	PTC	pT4N1bM0	I
57.	Female	54	PTC	pT4N1aM0	III
58.	Female	47	UTC	pT4N0M0	IV
59.	Male	77	UTC	pT4N1M0	IV
60.	Female	79	UTC	pT4N1M0	IV
61.	Male	82	UTC	pT2N0M0	IV
62.	Female	54	UTC	pT3N0M0	IV
63.	Male	75	UTC	pT2N0M0	IV
64.	Male	60	UTC	pT3N1M0	IV
65.	Male	68	UTC	pT3N0M1	IV
66.	Male	69	UTC	pT3N1bM1	IV
67.	Male	84	UTC	pT4N1M1	IV
68.	Male	35	UTC	pT3N0M1	IV
69.	Female	48	UTC	pT4N0M0	IV
70.	Male	60	UTC	pT3N1M0	IV
71.	Female	65	UTC	pT3N1M0	IV
72.	Male	43	UTC	pT3N1M0	IV
73.	Female	75	UTC	pT4N0M0	IV
74.	Male	52	UTC	pT4N0M0	IV
75.	Female	72	UTC	pT3N0M0	IV
76.	Male	62	Nodule goiter		
77.	Male	54	Nodule goiter		
78.	Female	75	Nodule goiter		
79.	Male	60	Nodule goiter		
80.	Female	68	Nodule goiter		
81.	Male	48	Nodule goiter		
82.	Female	60	Nodule goiter		
83.	Female	65	Nodule goiter		
84.	Male	42	Nodule goiter		
85.	Female	71	Nodule goiter		
86.	Male	68	Nodule goiter		
87.	Female	62	Nodule goiter		

Ethical approval

This Study involving human tissues described were approved by the local committees of medical ethics and all patients gave written consent.

3.1.8 Cell lines and cell culture conditions

Table 10: Cell lines and cell culture media used in this study

Cell type	Appellation	Source and Reference
Thyroid	FTC-133	supplied by Prof. P. Goretzki, was established from lymph node metastasis of a primary follicular thyroid carcinoma 90% DMEM/ F12+ 10% FBS ECACC 94060901
	B-CPAP	DSMZ, Braunschweig, Germany established from the tumor tissue of a 76-year-old woman with metastasizing papillary thyroid carcinoma 90% RPMI 1640 + 10% FBS
Colon	HT-29	DSMZ, Braunschweig, Germany established from the primary tumor of a 44-year-old Caucasian woman with colon adenocarcinoma in 1964 90% DMEM/ F12+ 10% FBS
	Caco-2	DSMZ, Braunschweig, Germany established from a primary colonic tumour in a 72-year-old Caucasian male 80% MEM (EBSS) + 20% FBS + 1% non-essential amino acids
	Lovo	DSMZ, Braunschweig, Germany established from the metastatic nodule resected from a 56-year-old Caucasian man with colon adenocarcinoma in 1972 90% RPMI 1640 + 10% FBS

Additions and solutions	Source
DMEM/ F12-medium	Gibco, invitrogen
Fetal bovine serum (FBS)	BioWest, Nuaille
Hank's Balanced Salts (HBSS)	Gibco, invitrogen
MEM	Gibco, invitrogen
L-Glutamin	Merck Eurolab GmbH
NaHCO3	Merck, Darmstadt
Penicillin / Streptomycin	Gibco, invitrogen
RPMI 1640	Gibco, invitrogen
Trypsin/EDTA solution	Gibco, invitrogen
Opti-medium	Gibco, invitrogen

3.2 Methods

3.2.1 Cell culture

Culturing, freezing and defrosting of cells

Culturing of cells in following steps:

Grow adherent cells to 90% confluence. Cells were washed once with 2 to 3 ml per 10cm dish of Trypsin/ EDTA Solution. 2ml of Trypsin/ EDTA Solution was added per 10cm dish. Incubate for 2min at 37º C till cells were seperated from bottom, then add 8ml of serum containing medium. Triturate the cells vigorously until a single-cell suspension is achieved.

Cells were counted in cell counter. Pellet the cells by centrifugation at 1000rpm in a table-top centrifuge (1,000× g) for 5 minutes. Aspirate the medium and resuspend the cell pellet in 10ml of medium. 1× 10^5 cells were seeded in a small size (25cm^2) culture bottle (5× 10^5 cells/ middle size culture bottle; 1× 10^6 cells/ large size culture bottle).

Freezing and defrosting of cells in following steps:

After trypsinization and centrifugation of cells, 5× 10^6 cells were resuspend in 1ml freezing medium [Fetal Calf Serum: DMSO (1:9)] and pipetted in a 2ml cryo-tube. Cells can be stored in liquid nitrogen for long time till using. Defrosting process of cells was performed according to a similar protocol as for cell generation. We defrosted the frozen cells with complete culture medium and transferred them to a fresh 50ml Falcon tube. After centrifugation, we aspirated the medium and resuspend the cell pellet in 10ml of medium. All of the cells (5× 10^6) were added in a middle size (75cm^2) culture bottle with 25ml medium.

3.2.2 Storage of cryo tissues and homogenization

Resected tissues were snap frozen in liquid nitrogen and maintained at -80º C until use. For homogenization of tissue sample we used a homogenizer. All tissue samples were separated into a part for RNA isolation and another part for cyrocut sections. Frozen sections at 6µm were cut on a cryostat and control sections were hematoxylin-eosin (HE) stained.

3.2.3 Extraction of total-RNA from tissue and cells

Total-RNA from tissue samples and cells was extracted using the TRIZOL reagent (Invitrogen) according to the manufacturer's protocol.

Trizol (1 ml) was added to 100 mg homogenized tissue powder or directly onto a 3.5cm diameter dish with cultured cells and lysates were passed through a pipette several times. The homogenized samples were incubated for 5min at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes.

Add 0.2ml of chloroform per 1ml of TRIZOL Reagent. Shake tubes by hand for 15 seconds and incubate them at room temperature for 2 to 3 min. Samples were centrifuged at 12,000× g for 15min at 4°C. After centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer the aqueous phase to a fresh tube. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5ml of isopropyl alcohol per 1ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at room temperature for 10min and centrifuge at 12,000× g for 10min at 4°C. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube. Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding 1ml of 75% ethanol per

1ml of TRIZOL Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 12,000× g for 5min at 4°C.
At the end of the procedure, briefly dry the RNA pellet (air-dry for 20min). Dissolve RNA in RNase-free water by passing the solution a few times through a pipette tip, and incubating for 10 min at 55°C and stored at -80°C.

3.2.4 Extraction of total-protein from cells

Total protein of cells was extracted with this lyses buffer. Insoluble components were removed by centrifugation at 15,000 rg for 45 min to remove the supernatant which contains the protein and place in a new eppendorf tube. An aliquot was saved to determine protein concentrations using the Bicinchoninic Acid method (BCA Protein Assay Kit) according to the manufacturer's instructions (Pierce, Number 23225). Final protein concentrations were adjusted to 2.0 mg/ ml and protein samples were stored at -80°C for later analysis.

3.2.5 Reverse Transcription Polymerase Chain Reaction

cDNA synthesis

Total RNA (1µg) was reverse transcribed to generate single stranded cDNA. Total volume 10µl with following inclusions was incubated for 3 min at 95°C.

- 1,0µg RNA (corresponding volume was calculated)
- xµl (=10-corresponding volume of 1,0µg RNA) DEPC-water

Within this 3 min the following reaction components were prepared and pipetted 15µl in each tube.

- 2,7µl DEPC-water
- 5,0µl 5× First Strand Buffer
- 2,5µl 0,1M DTT
- 3,0µl Random primers
- 1,0µl 12,5mM dNTP
- 0,3µl superscript II
- 0,5µl RNase out

Next steps of incubation were performed at 42°C for 45 min, at 95°C for 3min. The samples were stored at -20°C.

Amplification (PCR) and labeling of cDNA Fragments

The amplification program was performed within the linear range of both CD82 and 18S (the internal control) and consisted of 30 sec at 94°C, 30 sec at 60°C (18S: 62°C), 45 sec at 72°C (40 cycles for CD82 and 15 cycles for 18S), and a final elongation step for 7min at 72°C. For

simultaneous PCR amplification of CD63, ß-actin served as internal control. The amplification protocol consisted of 30 sec at 94°C, 30 sec at 56°C, 45 sec at 72°C for 30 cycles and a final elongation step for 7min at 72°C.

PCR mixture (each sample) containd:

- 16,8µl dH2O
- 2,5µl 10× PCR buffer
- 3,0µl dNTP mixture (100µM)
- 0,25 sense primer (10pmol/ml)
- 0,25 sense primer (10pmol/ml)
- 0.2µl AmpliTaq polymerase (5U/ µl)
- 2µl cDNA sample

Agarose gel Electrophoresis

PCR products (20µl) were run visualized under 50mA current in a 1% agarose gel plus ethidium bromide, photographed with Kodak Image System 440cf and electronically evaluated with Kodak Digital Science 1D-software (Eastman Kodak, New Heaven USA).

3.2.6 Evaluation of results

The expression of positive control was set as 100%. The expression levels of all investigated specimens were determined in comparison to the positive controls (100% human tonsil and HO cell line) and displayed in a colour grey scale as follows:

0%-25%	negative (-)
25%-50%	decreased (+)
50-75%	moderate expression (++)
≥ 75%	positive (+++)

3.2.7 Immunohistochemistry/Immunocytochemistry

Immunohistochemistry was performed using Dako cover plates. Freshly cut cryo-embedded serial 6µm sections of all tissues were incubated for 20min in a 1:4 mixture of 3% H_2O_2 in ice cold 90% Methanol. After two times washing with phosphate-buffered salt solution, sections incubated with normal swine serum (1:4 diluted) in 1% BSA (PBS-BSA) for 10min to suppress non-specific binding. Successive sections were incubated overnight at 4ºC with mouse monoclonal antibodies (mAbs) against human CD82 at the dilutions of 1:100 and the mAb against human CD63 at a dilution of 1:200, respectively. Negative control sections were exposed to the secondary antibody only and processed as described above.

After 3×10min washes in PBS, sections were incubated for 30min with a 1:1000 dilution of biotinylated goat anti-mouse secondary antibody followed by incubation with an avidin–biotin-

peroxidase complex. After three times washing in PBS, specific immunostaining was visualized with diaminobenzidine (DAB) chromogenic solution (1:50). Finally, sections were lightly counterstained with Mayer's hematoxylin. Human tonsil tissue sections were used as positive controls.

All immunostained tissue sections were examined by two independent reviewers. For better quantification of specific immunostaining, planimetric measurement of immunoreactive cell clusters and tissue parts was evaluated semi-quantitatively using an Axioplan light microscope (Zeiss, Jena, Germany) by two independent investigators blinded to the histological diagnosis. Planimetric evaluations on immunostained specimens were also performed with the Zeiss KS300 software and the plasma-nucleus relation of immunopositive cells was determined. A staining score was estimated by calculating the percentage of cells that had membrane staining for CD82 multiplied by a factor (0-3) reflecting the percentage of specific immunostaining intensity.

0%-10%	negative (0)
10%-50%	weak (1)
50-80%	distinct (2)
≥ 80%	strong (3)

3.2.8 Immunofluorescence Staining

One day before experiment, approximately, 5×10^4 of FTC-133 cells were plated per 24-well plate in 500µl DMEM media with 10% FCS. On the day of experiment, growth media were pipetted off from the well plate. Cells were washed twice with ice-cold PBS and fixed with 4% Formaldehyde in PBS buffer for 30 min. After the fixing formaldehyde fix was removed, cells were washed three times with PBS buffer (5min per wash), then incubated 1min with permealization solution. Saponin buffer was fresh prepared and filtered containing PBS, 1% heat inactivated FCS, 0.1% w/v sodium azide and 0.1% w/v saponin azide. Cells were incubated with primary antibody diluted in 0,1% PBS (dilution 1:200) for overnight at 4^oC in wet chamber. Second day, the primary antibody was removed and three times (10min per time) washed with PBS. Then blocked with blocking buffer (1:10 Donky serum in saponin puffer) for 10min and incubated with second antibody diluted in saponin buffer (dilution 1:200) for 1h at room temperature under the protection from light (cover with foil). After three times washing with blocking buffer, cells were incubated for 2 min in PBS with Hoechst (1:100). Finally, cells were washed twice (10min per time) with PBS and covered with mowiol solution for fixing the fluorescence.

3.2.9 Western Blot

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Reduced and non-reduced samples were prepared and proteins were separated using a SDS-PAGE gel. Appropriate percentage SDS-PAGE gel was used for protein of interest. Typically 12% acrylamide gels are used for high molecular weight (MW) proteins (>50 kDa), 15% gels for mid range MW proteins (15 - 50 kDa) and 20% gels for low MW proteins (<15 kDa). A 10% polyacrylamide-SDS gel with a 5% stacking layer was cast and run in a mini-Protean II device. To each lane, 20µg total protein in gel loading buffer (0.04% bromophenol blue) were added (1:1) proportionally. A mixture of 5µl High-Range Rainbow Molecular Weight Marker and of 5µl loading buffer was used. All samples were denatured at 95°C for 10 min and immediately on ice before loading onto the gel. Electrophoresis was performed at 40mA for about 2h at room temperature. Transfer to a polyvinylidene difluoride membrane (PVDF, amersham pharmacia biotech) was performed at 25V and 6^{o}C overnight using the wet mini-Transblot cell, according to manufacturer's instructions. The proteins were blocked with 5% nonfat dry milk in TBST buffer for 1.5h at room temperature, and then incubated with CD82 primary antibody at a 1:2,000 dilution (anti-β-actin antibody was diluted at 1:20.000) in working buffer overnight at 4^{o}C. The membranes were washed in TBST buffer three times (10min each time) and then incubated in horseradish peroxidase-conjugated anti-mouse IgG for 2h at room temperature.

ECL detection, film development and results evaluation

The membranes were washed in TBST buffer three times (10min each time). PVDF membrane was dipped in Pierce Western Blotting substrate Reagents for 1min. Immunoreactivity was detected and exposuring to X-ray film in dark room. Numeric data were obtained by densitometry scanning analysis using Kodak Digital science 1D software (Kodak Digital Science Electrophoresis Documentation and Analysis System 120).

3.2.10 Plasmid

A vector systern (pcDNA3.1, Invitrogen) was used in this experiment. This 5.4kb vector designed for high-level stable and transient expression in mammalian hosts.
It contains the following elements:

- human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells
- multiple cloning sites in the orientations to facilitate cloning
- neomycin resistance gene for selection of stable cell lines
- episomal replication in cells lines that are latently infected with SV40 or that express the SV40 large T antigen

CD82 cDNA coding sequence insert with the pcDNA 3.1 plasmid ligation process was established and kindly provided by Prof. Dr. Zhang XA in the Vascular Biology Center of the University of Tennessee, Memphis, USA.

3.2.11 E.coli culture

Upon transfection into E. coli, single colonies were picked from an LB-ampicillin plate and overnight cultures of these E.coli clones were grown overnight in LB (Luria-Bertani) medium (containing 1l H_20: 12g Trypton, 5g yeast -extract, 5g NaCl, 1g Glucose) und 50µg/ml Ampicillin.

3.2.12 Extraction of plasmid DNA by mini preparation Kit

Plasmids were isolated from E. coli cells (5mL overnight culture) using the QIAprep Spin Miniprep kit (Quiagen, Hilden) according to the protocol recommended by the manufacturer.

3.2.13 Stable eukaryotic transfection of CD82 construct

Transfection procedure

One day before transfection, approximately, 5×10^4 of FTC-133 cells were plated per 24-well plate in 500µl DMEM media with antibiotics but without 10% fetal bovine serum to achieve about 70% of confluence. Cells were incubated under their normal growth conditions (generally 37°C and 5% CO2) for 30h. Transfection of the plasmid-CD82 and empty-plasmid was performed using Lipofectamin 2000 and opti-medium. Optimization of the transfection procedure was achieved with different plasmid-DNA concentrations. The mixture was prepared in following procedure:

- A: for each well: 2µl Lipofectamine 2000 + 50µl Opti-medium (5min. incubation at room temperature)
- B: 1µg plasmid-DNA + 50µl Opti-medium
- A + B (incubation 20 min at room temperature)

The cells were washed once with pre-warmed (37°C) PBS. Then, each well received 400µl of DMEM medium with antibiotics but without 10% fetal bovine serum. Subsequently, the transfection mixture was added to each well and the incubation of transfection continued for 10hr at 37°C, 5% CO_2 in a humidified incubator. The transfection medium was replaced by normal growth medium (DMEM) containing 10% FBS and antibiotics.

Geneticin (500µg/ml) was used for the selection of transfectants after transfection. One day after transfection, selection medium replaced the normal medium and then was changed every 2 days to remove debris from dead cells. Geniticin-resistant cell transfectants were selectived and picked after 6 weeks of culture.

3.2.14 Selection of stable transfectants

To investigate the influences of CD82-plasmid on FTC-133 cells, after Geneticin selection (500µg/ml), three different clones were selected separately from transfectant cells. Positively identified stable FTC-133-CD82 transfectants were grown in the presence of geniticin selection.

3.2.15 Validation of CD82 overexpression

The overexpression of CD82 after transfection and selection was validated by RT-PCR and western blot analysis.

3.2.16 Cell vitality assay (MTT assay)

One day before cell seeding, culture medium was been replaced with medium without serum. 5×10^3 cells were seeded on the 96-well plate on the day of cell seeding. After 24h, 48h and 72h of seeding, the MTT assay will be performed in following procedures. Cultured cells were washed with prewarmed medium without serum, MTT working solution was added into wells being assayed (20µl/ well). Cells were incubated at 37°C for 4h. At the end of the incubation period, the medium with MTT working solution was removed from well plate. 100µl DMSO was added to each well. Pipetted up and down several times to make sure the converted dye dissolves completely. Finally, absorbance of the converted dye is measured at a wavelength of 570nm in Spectra Rainbow ELISA. Measurement data was analysed by using easyWin screening ELISA program.

3.2.17 Cell motility assay (Boyden chamber assay)

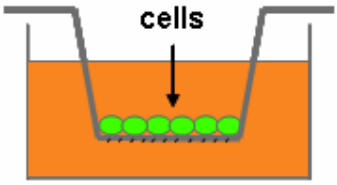


Figure 3: schematic construction of Boyden chamber assay

Transmigration assay were performed by using a modified Boyden Chamber system. Transwell chambers with polycarbonate membrane filters of 8 µm pore diameter were used to form an upper and lower compartment in a 24-well tissue culture plate. Before invasion assays, the invasion chambers were rehydrated with DMEM (serum free) for 2h in a humidified tissue culture incubator at 37°C with 5% CO_2 atmosphere. One day prior to the experiment, DMEM with 10% fetal bovine serum was added to the lower compartment. 1×10^4 cells what cells in serum-free DMEM were placed onto the filter and allowed to grow to confluence. Each cell group was plated in 3 duplicate wells. 24h after cell seeding, all of

polycarbonate membrane filters were washed briefly one time with cold PBS buffer. Then, cells were incubated for 5min in cold methanol and PBS solution (1:1 proportionally). The filters were dipped in pure methanol for 15 min. Finally, they were stained with toluidin blue for 30min and dried at RT for 2h.

The cells on the top surfaces of the filters were wiped off with a cotton stick. The cells that had penetrated the filter into the lower compartment were quantified. Pictures of 5 random visual fields (200×) per membrane were taken under a Zeiss light microscope and counted. Each experiment was performed in triplicate and repeated three times.

3.2.18 Stimulation experiment of FTC-133 cells with all-trans retinoic acid

The experiment was designed and divided into two groups: control group and RA (retinoic acid) group. 5×10^5 cells were seeded in middle size ($75cm^2$) culture bottle. The confluent cells were cultured in serum-free medium for 24h, and then cells were incubated with or without all-trans retinoic acid. Control group was treated with DMEM growth medium, RA group was stimulated with all-trans retinoic acid (concentration: 1 µmol/L). 24h, 48h and 72h after cell seeding, total mRNA of cells were harvested for analysis of endogenous CD82 mRNA expression.

3.2.19 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Isolated proteins were cleaned with 2-D Clean-Up Kit and the concentration was confirmed with 2-D Quant Kit. For Two-dimensional gel electrophoresis 30µg protein was dissolved in 450µl Rehydration Solution. Firstly, protein samples were loaded on 18cm Strip Holder and the Immobiline DryStrip gel (pH 3-10) was positioned. The Immobiline DryStrip gel overlayed with Immobiline DryStrip Cover Fluid, then the program was runned in the IPGphor II Isoelectric Focusing system. After running program, the Immobiline DryStrip gels were equilibrated in Equilibration buffer for 15min (75 mM Tris-HCl, pH 8.8). 12,5% Gel applyed and covered with 2- methyl- 1- propanol, Immobiline DryStrip gels were pushed into12,5% gel and covered mit 0,5% Agarose (0,5% Agarose: 1×SDS+ 0,5%Agarose+ 0,002% Bromphenol blue). Filling Ettan Daltsix electrophoresis chamber with anode buffer (Anodenpuffer 1× SDS: 25mM Tris, 192mM Glycine, 0,1% SDS) and position the gel cassette; filling Ettan Daltsix electrophoresis chamber with cathode buffer (cathode buffer 1× SDS: 25mM Tris, 192mM Glycine, 0,1% SDS). The gels were run at 20°C (power supply: 30 Min 2,5W pro Gel and overnight 4-5W pro Gel). Each experiment was performed at least in triplicate.

3.2.20 Silver staining

The gels were fixed in 400ml ethanol, 10% cold acidic acid and 1l monodest for 1h or overnight. After three times washing with 300ml ethanol diluted in 1l monodest the gels were sensitivated in 0,2g Natriumthiosulfat diluted in 1l monodest for 1min. Then the gels were washed three times (20sec per time) in monodest and stained with 5% silver nitrite solution, 250µl Formaldehyd (37%) diluted in 1l monodest for 20min. After three times washing with monodest, the gels were developed in 30g Natrium carbonate, 250µl Formaldehyd (37%) diluted in 1l monodest for 5min. The gels were washed once in monodest for 20sec and staining was stopped with 50ml acidic acid diluted in 1l monodest for 10min. Finally, the gels were washed three times with monodest (10sec per time).

3.2.21 Scanning and computer analysis of the 2-DE

After protein diffusion and staining, the gels are scanned by means of a special flat bed scanner. In the subsequent stage of automatic image analysis, the images are LP-filtered, background and protein regions are segmented and saturated regions identified. The final quantification and modeling of the protein spots may leads to parametric representations of each spot. Besides a compressed, quantitative description of the image content, they also provide a more suitable means for following studies such as spot matching between different gels.

In this study, we used the evaluation software “Phoretix 2D analysis software” (Biostep GmbH) to compare the different 2D gels. The Phoretix 2D software evaluates and compares spots between different 2D gels and a virtual gel in which each protein spot is averaged over a number of individual gels that were run with the same protein extract under similar conditions.

3.2.22 MALDI-TOF/ Q-TOF Mass spectrometrical analysis

For identification, quantification, and characterization proteins in wild type follecular thyroid carcinoma cells (FTC-133) and different transfectants, the Voyager-DE™ PRO Biospectrometry Workstation is a benchtop MALDI-TOF mass spectrometer designed to accurately determine molecular weights on subpicomolar quantities of molecules. The system is used for routine non-expert operation and incorporates Delayed Extraction™ technology for excellent sensitivity, mass accuracy, and resolution.

The QSTAR® XL Hybrid LC/MS/MS System is a high-performance, hybrid quadrupole time-of-flight mass spectrometer designed for proteomics research. The system generates superior quality MS and MS/MS data from both atmospheric pressure ionization (API) and matrix-assisted laser desorption ionization (MALDI) techniques. This unique flexibility to

switch between the standard API, NanoSpray™ source, and the new oMALDI™ 2 ion source makes the QSTAR XL system the preferred choice for proteomics. A specific scan mode such as precursor ion scanning, enabled by the patented LINAC™ Pulsar collision cell technology, and identifies the type and location of post-translational modifications with outstanding specificity and sensitivity.

Mass spectrometry (MS) is currently a key technology driving analytical proteomics. MS analyses of peptides generated by proteolytic digest provide information to identify the sequence and post-translational modifications of many proteins. Protein mixtures such as cell and tissue extracts, subcellular fractions and protein complexes are first subjected to some type of separation in order to resolve several fractions containing fewer components. Usually this approach employs two-dimensional SDS-PAGE in which proteins are separated according to their isoelectric point and molecular weight, respectively. Each spot on resulted 2D image represents a single protein with unique properties and can be analysed using MS technique. Selected spots containing proteins of interest are cut out of the gel, digested with proteolytic enzyme trypsin, desalted and subjected for two principal MS analyses- matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) and electrospray ionisation-tandem mass spectrometry (ESI-MS/MS). MALDI-TOF MS employs laser energy to generate peptide ions from co-crystallised mixtures of peptides and ultraviolet-absorbing organic acids. Masses of different peptide ions within one protein are displayed as TOF spectrum and can searched against different databases to identify the protein from which the peptides were generated. This technique, called peptide mass finger printing, allows easy and rapid identifying of proteins for which the sequence databases are available. However, this technique becomes difficult in higher organisms for which the genome and protein sequences are unknown. These problems can be omitted applying ESI-MS/MS technique, here the peptides are ionised under high voltage at atmospheric pressure and then subjected to ion trap or triple quadrupole mass analyzers. MS/MS analyses include two step processing. First, the masses of peptides from selected spot are resolved using TOF mode and resulting TOF spectrum with all peptide finger prints can be searched against data bases in order to identify the protein or analyse in product ion mode. Second, all multiply charged peptides observed in TOF mode are fragmented in collision cell (product ion mode). These fragmentation patterns are recorded in the resulting MS/MS spectra, which provide finger prints that define the sequence of peptide ions and aminoacid sequence of analysed peptide. Performing fragmentation and sequencing of all peptides obtained from one spot will allow the identification and sequence of whole proteins. Moreover, considering the spectral characteristics of some peptides (appearance or loss of typical residues), this technique is useful in mapping of post-translational modifications. MS spectra were sent to MASCOT (http://www.matrixscience.com) for final database search.

3.2.23 Statistical analysis

Microsoft Excel and Sigmaplot 8.0 were employed for all graphs calculations. The statistical significances of the experimental and clinical-pathological parameters were calculated using the proportional t-test and the differences between groups were determined by the Mann-Whitney-Wilcoxon-test (U test) features of SPSS 10.0 software for Windows. P-values of < 0.05 were considered to indicate statistical significance.

4 RESULTS

4.1 CD82/ CD63 expression in human thyroid tissues

4.1.1 Transcriptional analysis

CD82

All investigated benign goiter tissues showed strong transcriptional level for CD82. Thyroid carcinoma tissues displayed lower CD82 mRNA expression. Out of the 36 primary thyroid tumors studied, 24 (66, 7%) revealed moderate or strong expression of CD82, whereas 12 (33, 3%) had decreased CD82 mRNA levels or did not express CD82 transcripts. By contrast, all of the 11 investigated distant metastases did not express CD82 mRNA and 17 out of 28 (60, 7%) regional lymph node metastases displayed significant decrease or loss of CD82 transcription. CD82 mRNA expression revealed a significant correlation between different pN (pN0 versus pN1: p< 0,05) and M (M0 versus M1:p=0.08) status of differentiated and undifferentiated thyroid carcinoma and the pathological staging of differentiated carcinoma (SI,II versus SIII: p=0.221; SIII versus SIV: p< 0,05; SI,II versus SIV: p< 0,05) but no correlation with age, gender and tumor size (Table 11, and in Figure 5: a-e).

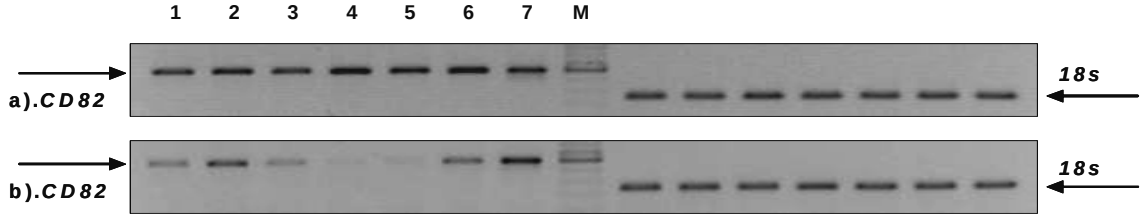


Figure 4: Agarose gel electrophoresis of RT-PCR analysis of freshly resected tissue samples. Each panel indicates quantitation of the CD82 RT-PCR products normalized to the level of 18S mRNA. (Lane 7: positive control. M: molecular marker). a) the entire benign thyroid goiter showed positive CD82 mRNA expression (Lane 1-6); b) Absent or significantly reduced CD82 expressions in mRNA from tumor tissues are evident in 6 thyroid carcinoma patients with different histopathological diagnosis.(Lane 1 and 3 pT2N1M0, pT3N1M0; Lane 2 and 6: pT1N0M0, pT3N0M0; Lane 4 and 5: pT3N1M1, pT4N1M1.)

The following box plot diagrams clarify the correlations between CD82 mRNA expression and various clinical/pathological factors.

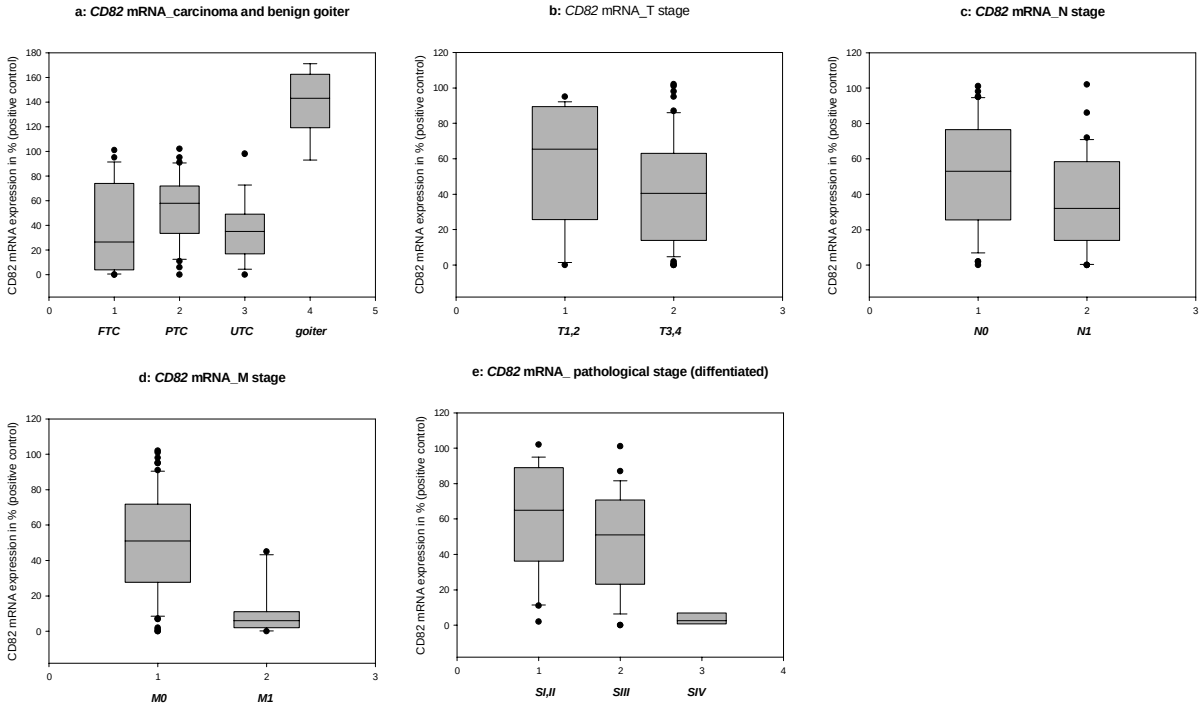


Figure 5: a-e: CD82 mRNA was detected in all goiter tissues and expression was determined as strong. Significant lower CD82 mRNA expression was detected in thyroid carcinoma tissues than in benign goiter tissues. No significance was determined between differentiated and undifferentiated thyroid carcinoma (a). No significant differences in the CD82 mRNA expressions were statistically evaluated between pT1/ pT2 (n=16) pT3/ pT4 (n=59) (b). N0 (n=40) /N1 (n=35) and M0 (n=64)/ M1 (n=11) stages showed significances (c, d). Stage IV differentiated tumors (n=11) revealed with significant higher CD82 mRNA expression than stage I,II (n=21) and stage III (n=29) differentiated tumors (e).

CD63

All investigated carcinoma and benign goiter tissues contained transcripts encoding for CD63. CD63 transcriptional levels were higher in FTC tissues when compared to goiter tissues ($p<0.001$). No statistical significant correlation was observed between CD63 expression and age, gender, histological subtype, tumor size, the presence of lymph node metastases, distant metastasis and pathological staging. The relationship between CD63- mRNA expression and various prognostic factors is shown in Figure 7: a-e.

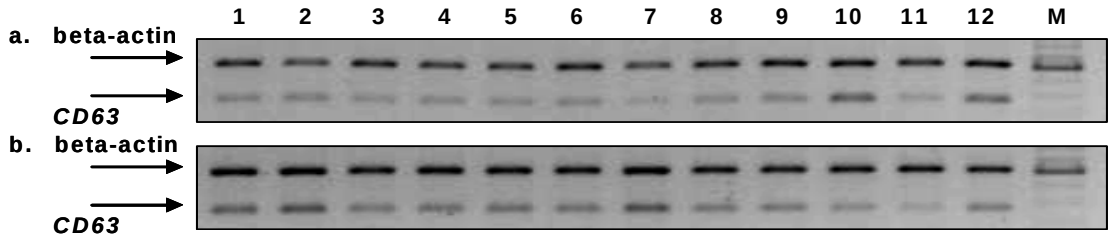


Figure 6: Agarose gel electrophoresis of RT-PCR analysis of resected tissue samples.
Each panel indicates quantification of the CD63 RT-PCR products normalized to the level of ß-actin mRNA. (Lane 12: positive control. M: molecular marker). a: All of the benign thyroid goiters showed positive CD63 mRNA expression (Lane 1-11). b: All of the thyroid carcinomas displayed positive CD63 mRNA expression (Lane 1-11).

The following box plot diagrams demonstrate the correlation between CD63 mRNA expression and various clinical/ pathological factors.

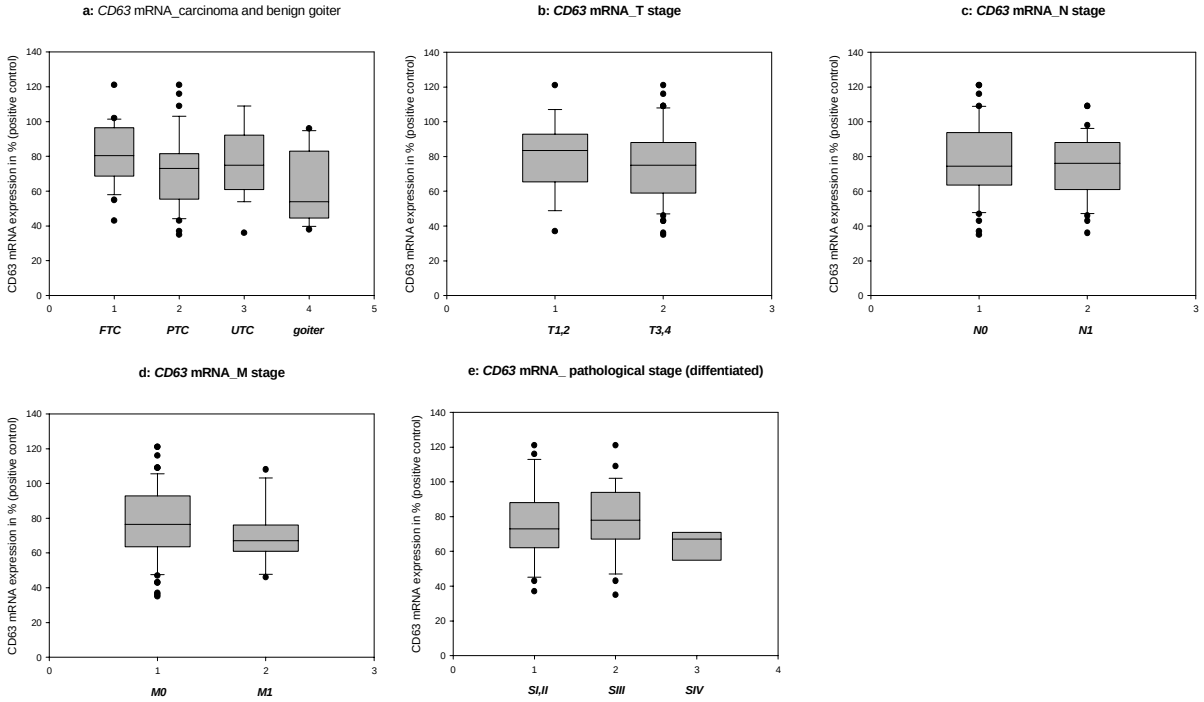


Figure 7: a-e
All carcinoma and benign goiter tissues were evaluated as CD63 positive. However, CD63 expression was significant higher exclusively in FTC as compared to goiter tissues (a). No statistically significant correlation was observed between CD63 expression and tumor size, the presence of lymph node metastases, distant metastasis and staging (b, c, d, e).

4.1.2 Immunohistochemistry

CD82

In benign goiter tissues and in normal human tonsilar tissues (squamous epithelium and lymphocytes), intense granular membrane-associated CD82 immunostaining was detected. Exemplary samples are shown in figure 7: a-h. Of the thyroid carcinoma tissue specimens studied, 21 out of 36 (about 58%) primary tumors showed moderate to high CD82 immunostaining. By contrast, 23 out of 28 (82%) primary tumors with regional lymph node metastases showed absent or reduced CD82 immunostaining, and all 11 distant metastases were immuno-reactive negative for CD82 (Figure 9:a-e). In agreement with the RT-PCR data, CD82 immunostaining showed a close correlation with (a) the N (N0 versus N1:p=0.008) and M status (M0 versus M1:$p<0.001$) of differentiated and undifferentiated thyroid carcinoma and (b) the pathological staging of differentiated carcinoma (SI,II versus SIII:p=0.142; SIII versus SIV:p=0,14; SI,II versus SIV:p=0.005). No correlation was observed for other clinico-pathological features such as age, gender or tumor status.

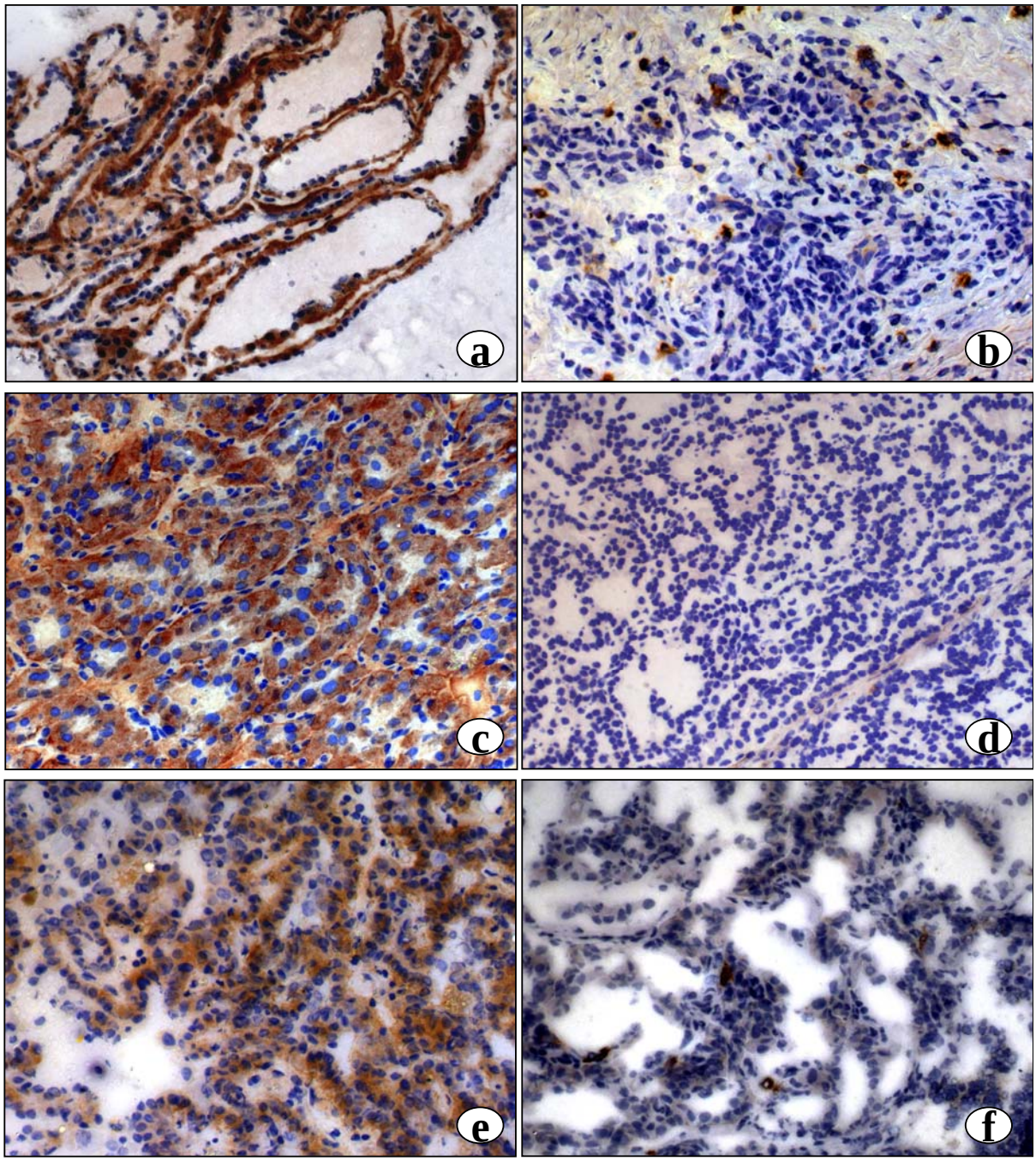


Figure 8: CD82 immunohistochemical staining patterns of frozen human tissues. **a,** benign thyroid goiter tissue, with a strong cell membrane reactivity expression of CD82; **c, e,** primary FTC and PTC (without regional lymph nodes metastasis and distant organ metastasis) showed CD82 protein expression strongly; **b, d, f,** weakly or negative CD82 protein expression in primary UTC, FTC and PTC (with regional lymph nodes metastasis and/ or distant metastasis).

The following box plot diagrams demonstrate the correlations between CD82 protein production and various clinical/ pathological factors.

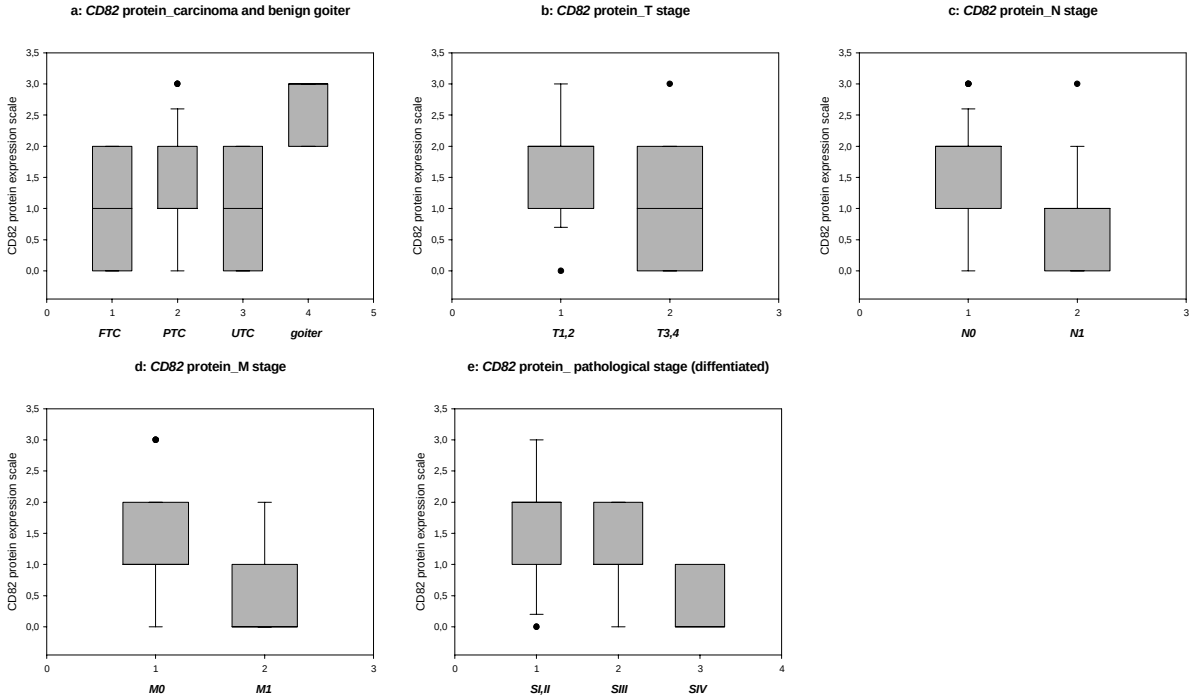


Figure 9: a-e
Detection of CD82 immunoreactive protein was successfully detected in the goiter tissues and production was determined as strong. CD82 tissue production was significantly higher than in thyroid carcinoma tissues. No production significance was determined between differentiated and undifferentiated thyroid carcinoma (a). No significant differences in the CD82 protein prodution were found between pT1/ pT2 (n=16) pT3/ pT4 (n=59) (b). Either N0 (n=40) /N1 (n=35) or M0 (n=64)/ M1 (n=11) stages (c, d), SIV differentiated tumors (n=11) revealed significant by higher CD82 mRNA expression than SI,II (n=21) and SIII (n=29) differentiated tumors (e).

CD63

Immunoreactive CD63 was detected in all thyroid tissues investigated. No statistically significant correlation was observed between CD63 immunoreactive protein expression and age, gender, histological subtype, tumor size, the presence of lymph node and distant metastasis or tumor staging.

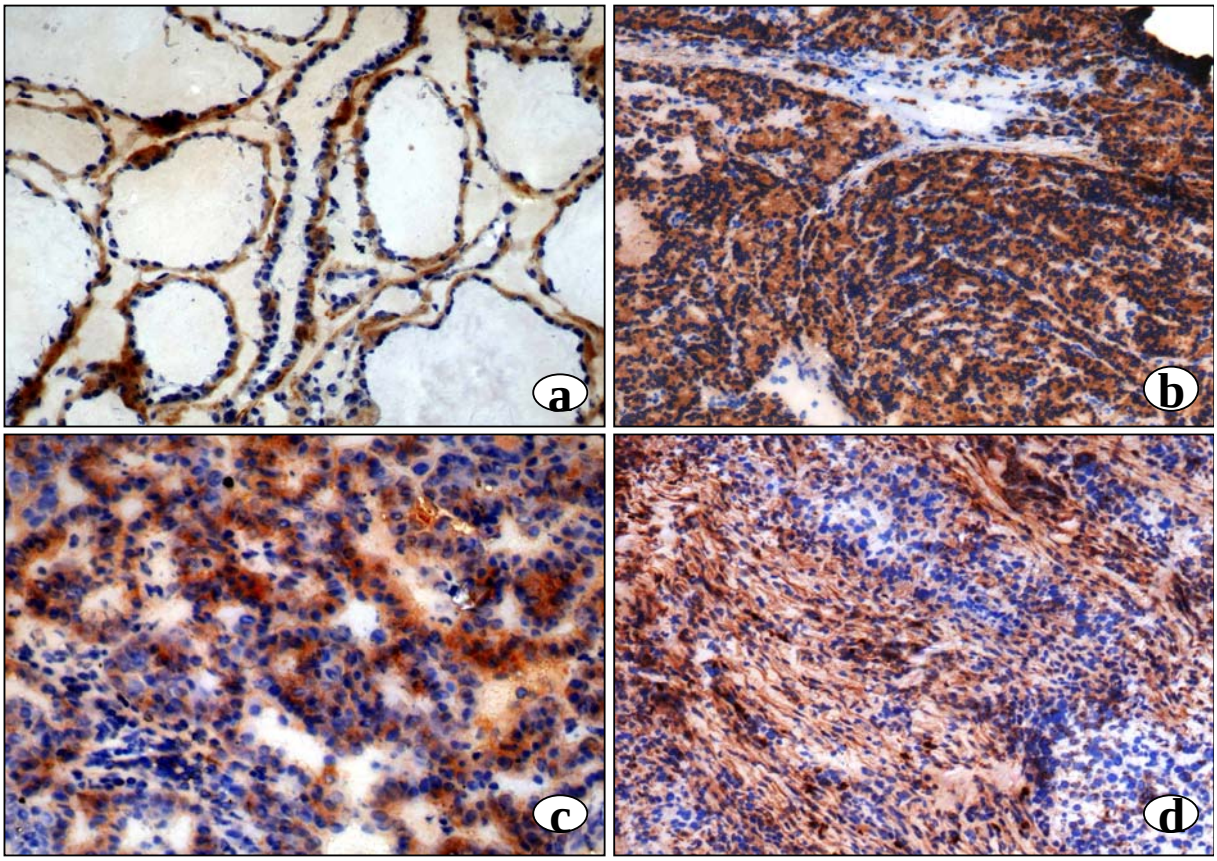


Figure 10: CD63 immunohistochemical staining pattern of frozen human tissues. **a,** benign thyroid goiter tissue, with a strong CD63 in cell membrane immunostaining; **b,** uniformly intense staining of CD63 protein in a primary FTC (pT4N1M0) with regional lymph nodes metastasis; **c,** a primary PTC (pT4NoM0) showed strong immunodetection CD63 protein; **d,** moderate staning in a UTC (pT4N1M0).

The following box plot diagrams demonstrate the correlations between CD63 protein production and various clinical/ pathological factors.

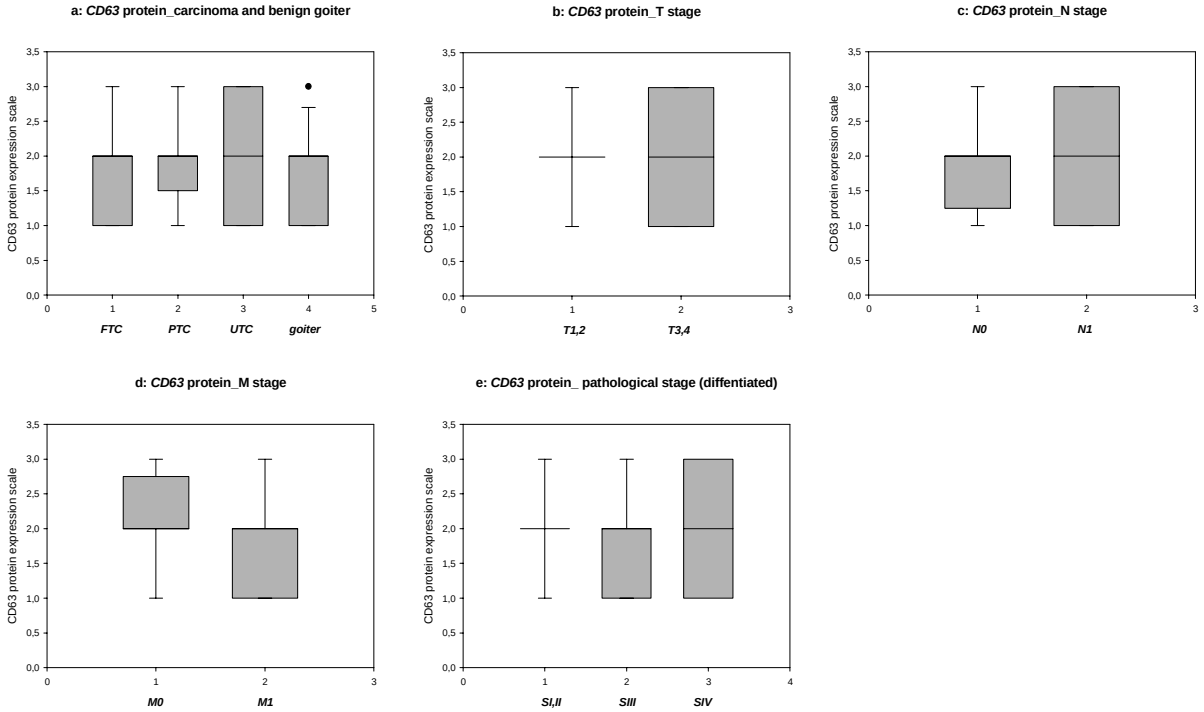


Figure 11: a-e
CD63 protein was detected in most investigated tissues (a). No statistically significant correlation was observed between the presence of immunoreactive CD63 and tumor size, the presence of lymph node metastases or distant metastasis and staging (b, c, d, e).

4.2 Summary of expression data

The relationship between CD82 expression and clinical pathological parameters is shown below.

Table 11: Relationship between CD82 expression and various clinical/pathological factors in 75 patients with thyroid carcinoma

Clinico-pathological characteristics	Patient Nr. (Total)	mRNA positive (++, +++)	mRNA reduced (-, +)	P-value	Protein positive (2, 3)	Protein reduced (0, 1)	p-value
Age (years)							
≤ 45	17	11	6		10	7	
> 45	58	23	35	NS	18	37	NS
Gender							
Male	29	9	20		8	21	
Female	46	27	19	NS	20	26	NS
Tumor status							
T1, T2	16	10	6		9	7	
T3, T4	59	25	34	NS	19	40	NS
Nodal status							
N0	40	24	16		23	17	
N1	35	12	23	0.023	5	30	0.008
Metastatic status							
M0	64	36	28		26	38	
M1	11	0	11	0.008	2	9	<0.001
Pathological stage (DTCs)							
Stage I, II	21	15	6	NS	12	9	NS
Stage III	29	17	12	0.027	10	19	NS
Stage IV	7	0	7	0.002	1	6	0.005
Total number of patients	75	32	43		28	47	

- NS, not significant.
- DTCs: differentiated thyroid carcinomas

4.3 CD82 expression in other human tissues

CD82 is ubiquitously strong expressed normal epithelium of human gastric mucosa and in squamous epithelium of tonsil tissue. In normal human tonsil gland and lymphoid tissue expressed also strong CD82.

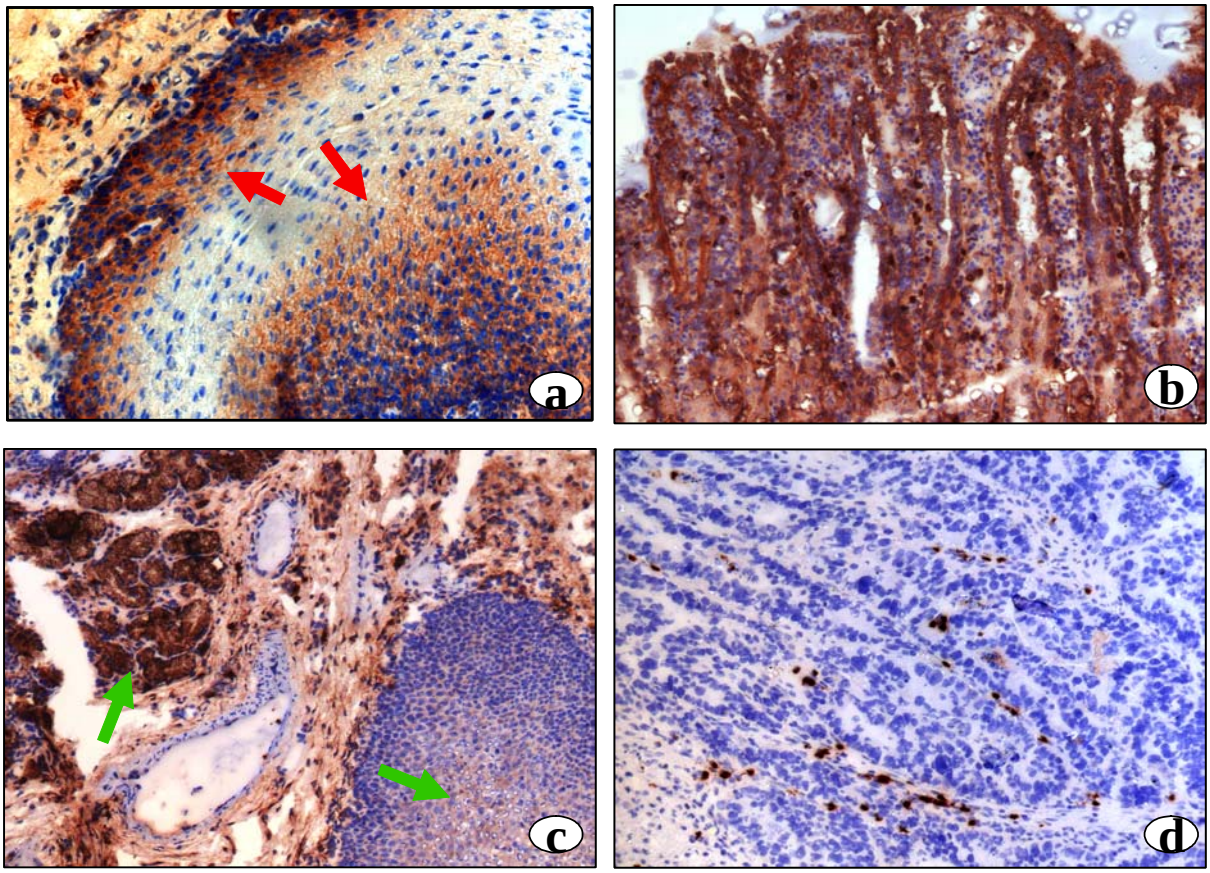


Figure 12: Immunohistological localization of CD82 in the normal epithelium of human gastric mucosa and external lining of stratified squamous epithelium of tonsil tissue (red arrow in a). In normal human tonsil gland and lymphoid tissue expressed CD82 strong ((green arrow in c). In a moderate differentiated gastric adenocarcinoma tissue cd82 was very weak expressed. Six-micrometer cryostat sections were stained using the ABC kit with diaminobenzidine as chromogen, and counterstained with hematoxylin-eosin. Anti-CD82 antibody (clone, C33: mouse IgG2a).

4.4 Characterization of CD82 overexpression in stable FTC-133-CD82 transfectants

Detected CD82 overexprssion at the transcript and protein level by using RT-PCR and Western blot were displayed below.

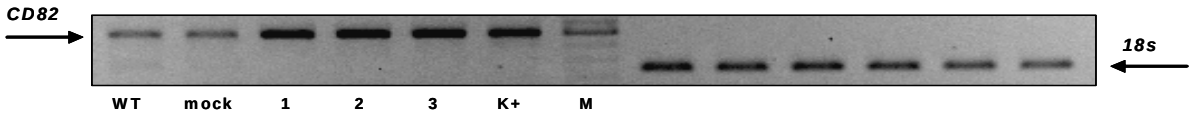


Figure 13: Overexpression of CD82 at transcript level: To the left side of the marker “M” are RT-PCR amplicons of CD82 transcripts (size 598 bp) from WT: wild type FTC-133 mock: empty plasmid transfectant 1, 2, 3: clone 1, 2, 3 FTC-133-CD82 transfectans. Clones 1-3 of FTC-133-CD82 show a significantly higher expression of CD82 in comparison to the WT and mock. On the right side of the marker are amplicons of the corresponding housekeeping gene 18S to confirm the presence of equal cDNA loading per RT-PCR reaction.

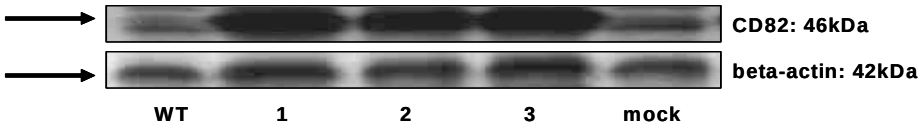


Figure 14: Western Blot: Overexpression of CD82 at the protein level in all CD82 transfected clones which showed strong CD82 protein expression (46KDa). Weak CD82 production was detected in WT and mock cells. WT: wild type FTC-133; mock: empty plasmid transfectant; 1, 2, 3: clone 1, 2, 3 CD82 transfectans, corresponding ß-actin (42KDa) demonstraed equal protein loading per lane.

4.4.1 Endogenous gene expression

The expression of endogenous MT1-MMP, S100A4, and VEGFc were decreased in overexpressed CD82 cells. No alterations in CD63, survivin (an anti-apoptotic factor) and TGF-ß1 as well as house keeping gene 18S expression could be found in transfectants in comparison with WT cells, but the upregulated Integrin α3 expression was observed in all transfected clones in comparison with WT and mock cells.

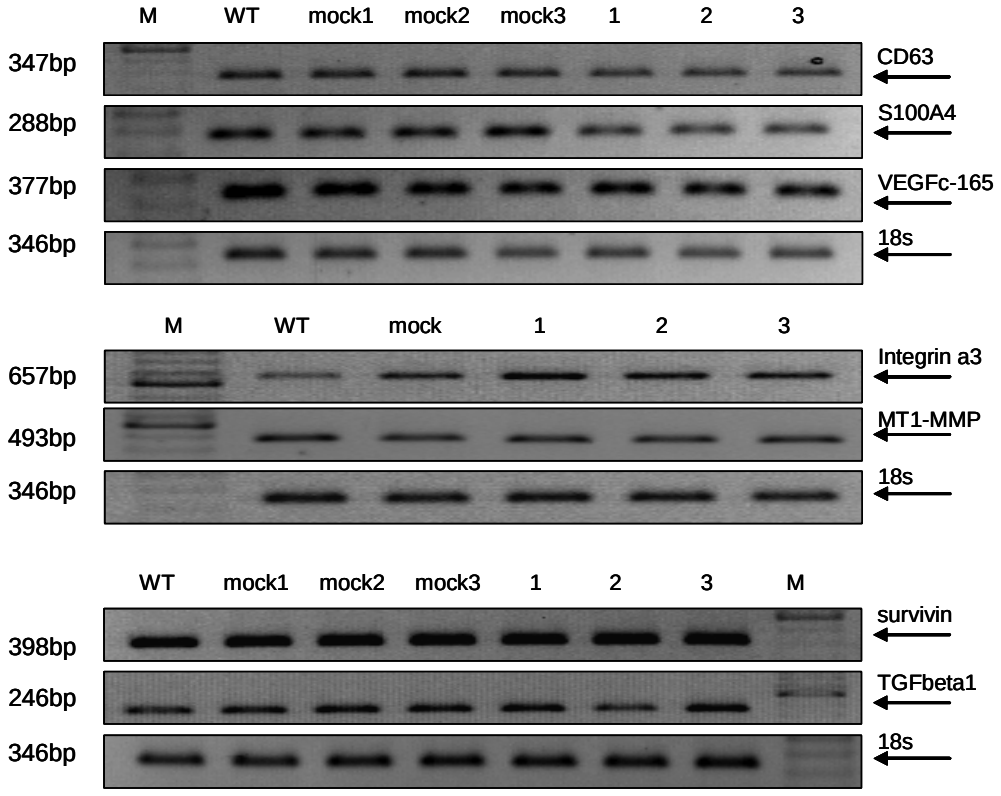


Figure 15: mRNA expression of CD63, S100A4, VEGFc, Integrin α3, MT1-MMP, survivin and TGF-ß1 in wild type and transfectants. (M: DNA ladder; WT: wild type; mock: empty plasmid transfectants; 1, 2, and 3: CD82 transfectants)

4.4.2 Cellular immunofluorescence staining

Cellular immunoreactivity of CD82 in wild type FTC-133 cells and FTC-133-CD82 transfectants was detected by using immunofluorescence staining. A strong CD82 immunoreaction in cell membrane and cytosol of transfectans cells can be observed (Figure 16), in comparison a very weak immunostaining in wild type cells.

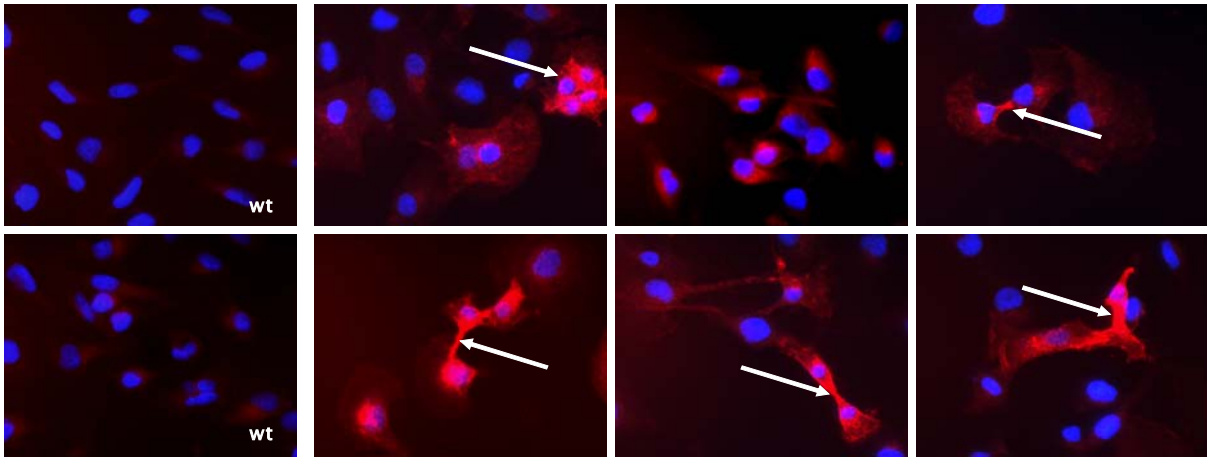


Figure 16: Immunofluorescence staining of CD82 in FTC-133 cells. Strong CD82 expression indicates a strong cell-cell-contact (white arrows). (wt: wild type)

4.4.3 CD82 mRNA expression in FTC-133 cells after RA treatment

24h, 48h and 72h after incubation with “empty medium” (serum-free) and empty medium plus all-trans retinoic acid, total RNA from cells were harvested for RT-PCR analysis.Compared to control group (no RA treatment), RT-PCR results showed the upregulation of endogenous CD82 mRNA expression induced by retinoic acid.

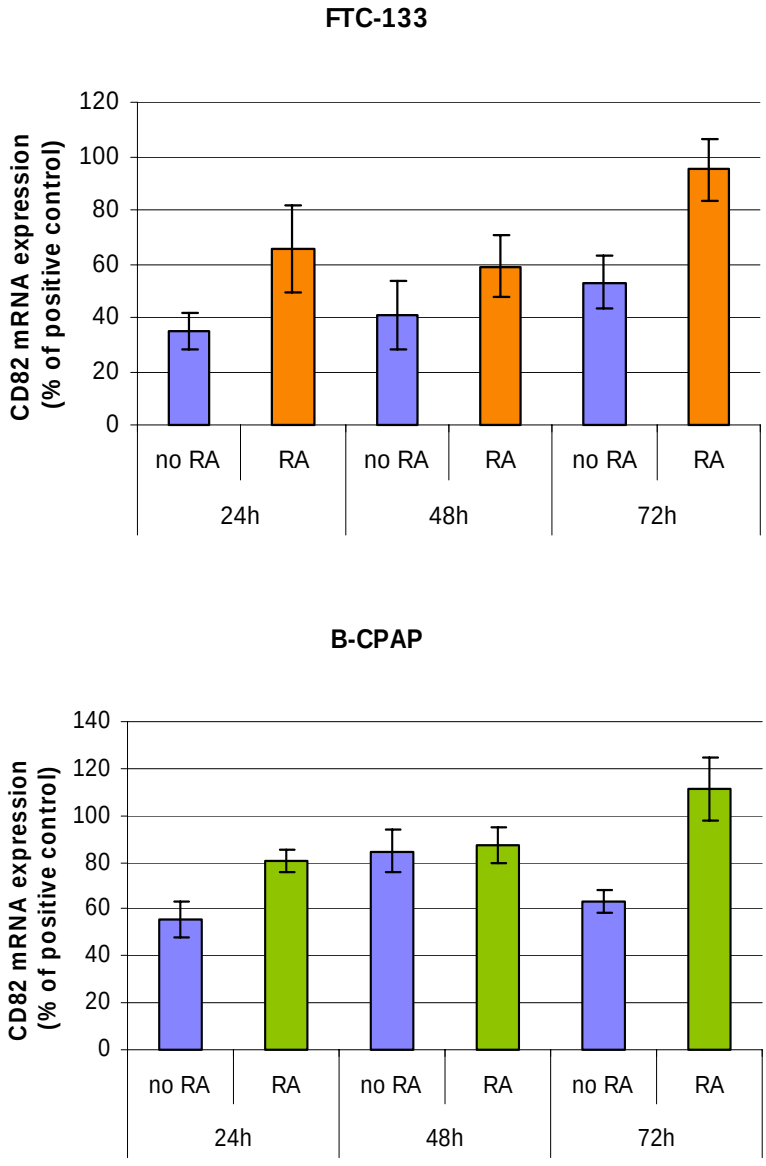


Figure 17: CD82 mRNA expression in FTC-133 and B-CPAP cells after RA treatment. The significant upregulation of CD82 mRNA expression were induced after all-trans retinoic acid treatment. Blue columns displayed the CD82 mRNA expression of untreated cells, and orange/green columns showed the CD82 mRNA expression of treated cells.

4.4.4 Cell vitality

Cell viability was assessed with the MTT assay. The measurement at 24hrs, 48hrs and 72hrs were displayed in blue, amaranth and yellow columns, respectively. Clone 2 showed a lowest cell viability among all investigated samples (0,31; 0,54; 0,6); clone 1 and clone 3 exhibited also lower cell viability than wild type and mock-transfected cells (0,32; 0,55; 0,67 and 0,34; 0,53; 0,71).

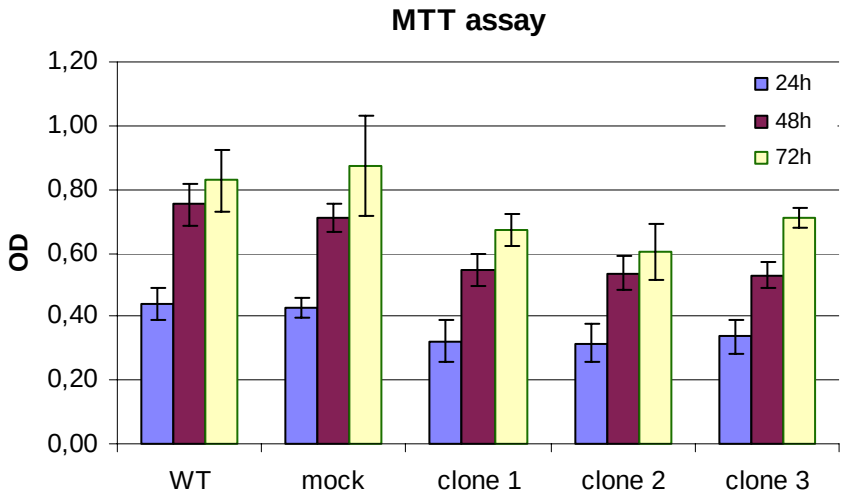


Figure 18: Comparisons of cell viability among wild type FTC-133 cells, mock transfected and three CD82 transfected FTC-133 cells.

4.4.5 Cell motility

In vitro invasive ability was tested by employing the Boyden Chamber assay. Cells migrating through the 8µm pores to the lower side of the filter were photographed and counted under a bright field light microscope. Figures 17 and 18 demonstrate the number of migrated cells of wild type FTC-133, mock transfected and CD82 transfected FTC-133 24h after seeding the cells onto filters. Fewer FTC-133-CD82 transfectants (clones 1, 2, 3) were detected at the underside of the filter as compared to mock-transfected or wild type FTC-133 (P<0.01).

Boyden Chamber assay showed that the cells penetrated the polycarbonate membrane filters in CD82 transfected (clone 1, 2, 3) were fewer than in wild type cells (P<0.01). However, the penetrated cell number of mock cells was almost unchanged than the wild type.

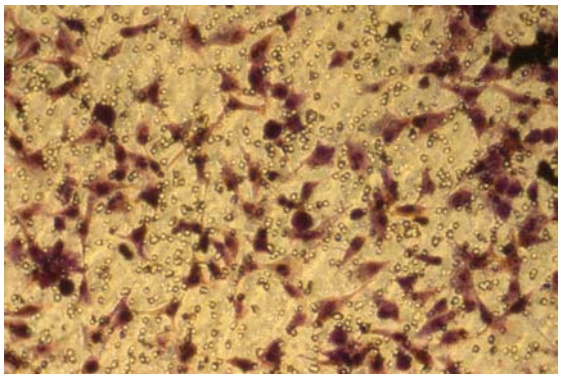


Figure 19: Underside of a filter after 24h migration of cells (brown) in a Boyden Chamber motility assay. (Giemsa staining, magnification ×200)

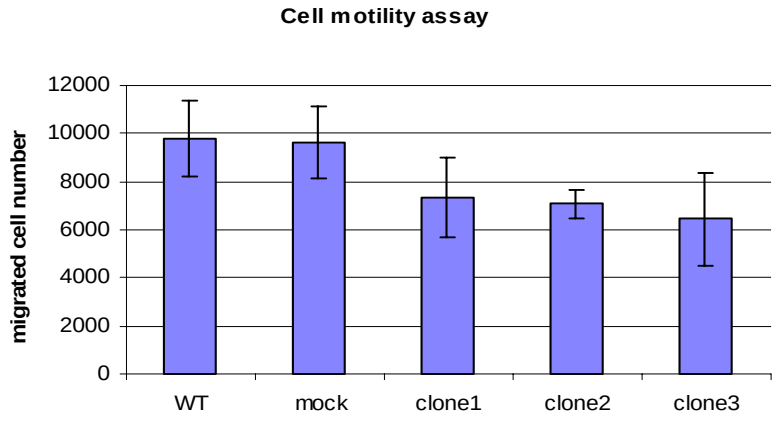


Figure 20: Cellular motility was determined by modified Boyden Chamber assay. Through 8 µm diameter pore filter migrated cells were counted under microscope.

4.4.6 Protein spots analysis and Phoretix 2D analysis

Silver stained polyacrylamide gels were performed and displayed the most protein spots of samples. For spot comparisons among the wild type cells, mock transfected cells and three transfectant clones showed following figures a general overview.

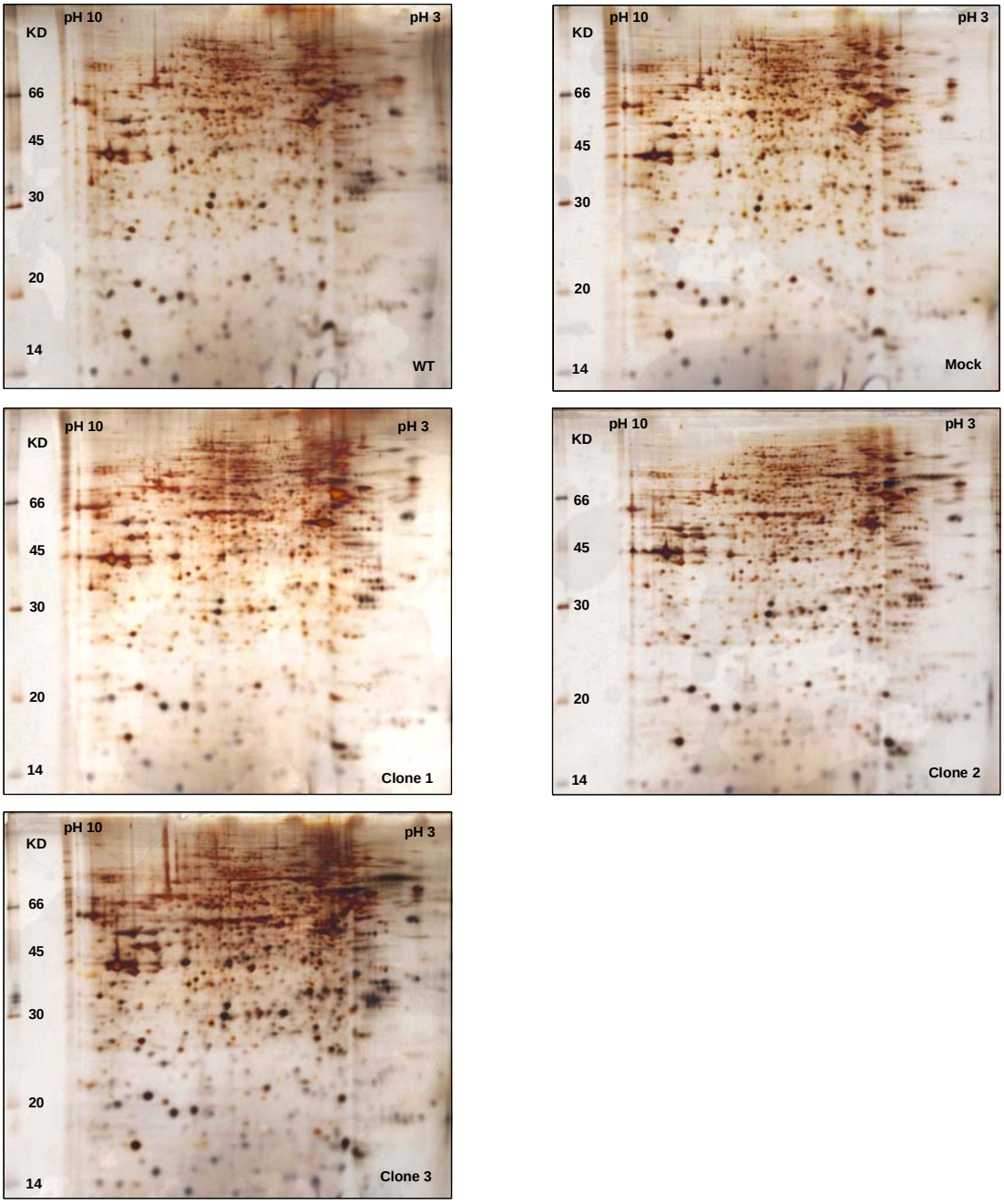


Figure 21: Silver stained polyacrylamide gels of isolated proteins from wild type FTC-133 and transfectants.

Phoretix 2D analysis

2D gel of wild type FTC-133 was set as reference gel and was compared to the mock transfectant and three different clones. The following data showed the results:

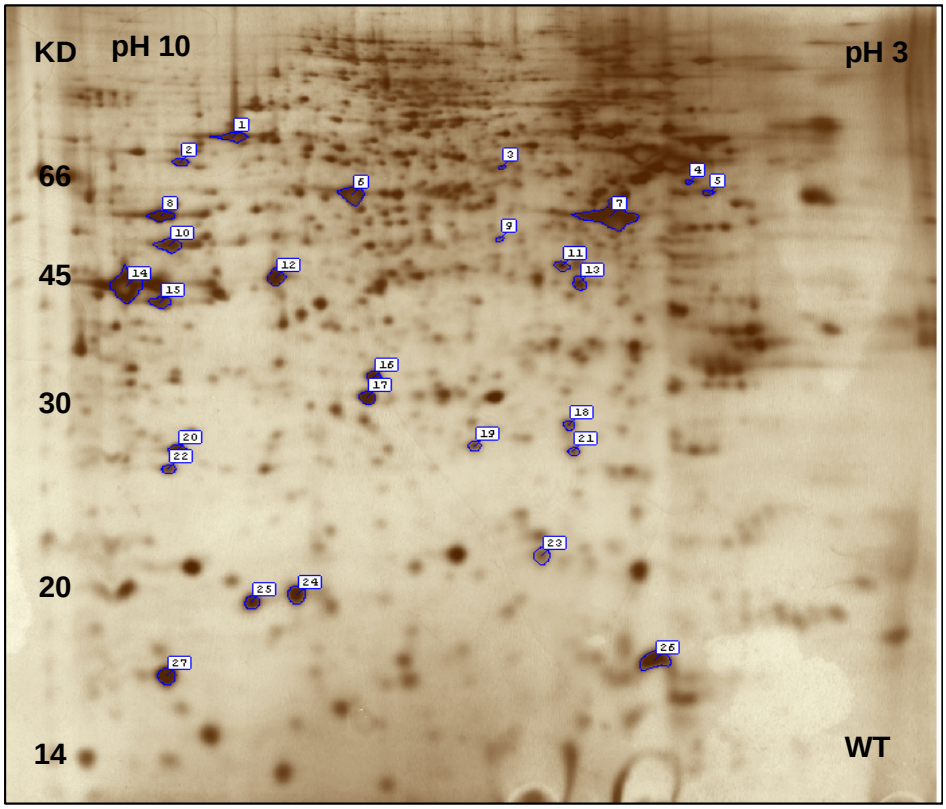


Figure 22: Reference gel with the reference spots of wild type FTC-133 cells after silver staining.

Table 12: scales of spots evaluation among the different gels: +: upregulated/ -: downregulated protein production; more than “+2 or -2” was considered as significant.

			Ref. Spot	FTC-133 mock			clone 1			clone 2			clone 3		
Protein	**Mr in kDa**	**pI**	**Ref. Spot**	**Norm. Vol.**	**Peak Height**	**Area**	**Norm. Vol.**	**Peak Height**	**Area**	**Norm. Vol.**	**Peak Height**	**Area**	**Norm. Vol.**	**Peak Height**	**Area**
Pyruvate kinase, M1 isozyme (Cytosolic thyroid hormon), human	57,769	7,95	**1**	2,07	2,033	1,089	1,445	1,295	1,253	-2,156	-1,054	-2,046	-1,175	-1,224	1,429
ATP Synthase, mitochondrial F1 complex, alpha subunit, human	59,772	9,07	**2**	-1,075	-1,054	1,036	-1,863	-1,778	-1,412	-2,723	-1,537	-1,901	-2,574	-2,574	1,276
Protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor, human	56,664	6,10	**3**	2,336	2,096	1,512	4,028	2,08	1,814	1,072	1,207	-1,344	1,235	-1,054	1,395
Vimentin, human	53,522	5,06	**4**	-1	1,217	1	-1,05	1,243	-1,735	12,053	2,544	3,847	-1,221	-1,146	1,085
Vimentin, human	53,522	5,06	**5**	-1,284	1,694	-1,603	2,361	2,355	-1,041	-4,066	-1,421	-3,061	1,233	1,201	1,139
Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase),human	47,008	6,99	**6**	1,505	1,716	-1,036	-1,408	-1,305	1,083	1,957	1,394	1,151	-1,047	-1,044	1,389
Actin, beta, Homo sapiens	40,978	5,56	**7**	1,332	1,558	-1,05	-1,844	-1,499	-1,161	-2,663	-1,622	-1,633	-2,168	-1,621	-1,022
Phosphoglycerate kinase, human	44,586	8,30	**8**	1,211	1,716	-1,32	2,176	1,632	-1,018	3,35	1,95	1,597	2,184	-1,041	2,574
Alpha enolase, human	36,286	6,53	**9**	1,829	2,191	1,183	1,404	1,451	-1,277	5,225	2,877	2,067	4,501	1,972	3,1

Protein	Mr in kDa	pI	Ref. Spot	FTC-133 mock			clone 1			clone 2			clone 3		
			Ref. Spot	Norm. Vol.	Peak Height	Area	Norm. Vol.	Peak Height	Area	Norm. Vol.	Peak Height	Area	Norm. Vol.	Peak Height	Area
Fructose-bisphosphate aldolase A, human	39,264	8,39	**10**	-1,853	-1,2	-1,497	-1,201	-1,324	-1,229	-2,004	-1,262	-1,811	-8,416	-4,478	-1,68
Tubulin alpha-6 chain (Alpha-tubulin 6), human	49,863	4,96	**11**	-1,208	1,176	-1,473	-1,459	-1,235	-1,838	-1,198	-1,043	-1,593	-2,172	-1,79	-1,493
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), human	35,899	8,58	**12**	-1,566	-1,062	-1,178	1,421	1,06	1,099	-1,227	1,017	-1,185	1,43	1,153	1,395
Tubulin beta-5 chain, human	49,639	4,78	**13**	-1,121	1,374	-1,145	-1,427	-1,118	-1,614	-28,115	-2,748	-12,182	1,068	-1,063	1,142
Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37), human	35,509	8,92	**14**	-1,518	-1,192	-1,151	-1,428	-1,464	1,005	-2,551	-1,919	-1,263	-2,369	-1,817	1,17
Galectin-3 (Galactose-specific lectin 3, MAC-2 antigen, IgE-binding protein), human	26,041	8,61	**15**	-2,751	-1,775	-1,292	-1,349	-1,221	-1,15	1,236	1,134	1,09	-1,281	-1,685	1,472
Phosphoglycerate mutase (EC 5.4.2.1) B (validated), human	28,786	6,67	**16**	-1,079	1,351	-1,048	1,077	1,089	-1,096	1,246	1,205	1,019	1,395	1,075	1,593
Triosephosphate isomerase (EC 5.3.1.1, TIM),	26,522	6,51	**17**	1,327	1,663	1,105	1,997	1,367	1,373	1,472	1,146	1,22	2,092	1,209	2,318

Protein	Mr in kDa	pI	Ref. Spot	FTC-133 mock			clone 1			clone 2			clone 3		
			Ref. Spot	Norm. Vol.	Peak Height	Area	Norm. Vol.	Peak Height	Area	Norm. Vol.	Peak Height	Area	Norm. Vol.	Peak Height	Area
Glutathione transferase pi (validated),human	23,341	5,43	**18**	-2,383	-1,102	-1,839	-1,048	-1,104	-1,14	1,293	1,287	-1,089	2,163	1,433	1,713
DJ-1 protein, human	19,834	6,33	**19**	-1,959	-1,03	-1,576	-1,282	-1,159	-1,397	-3,725	-1,643	-2,897	-1,262	-1,055	-1,053
Peroxiredoxin 1, human (QSTAR)	22,339	8,18	**20**	1,081	1,36	1,116	-1,84	-1,239	-1,734	-1,17	1,17	-1,297	1,029	1,184	1,094
Peroxiredoxin 2 (Thioredoxin peroxidase 1), human	21,878	5,66	**21**	-1,185	1,081	1,07	-1,259	-1,186	-1,327	1,813	1,477	1,042	2,131	1,261	1,683
Transgelin 2, human	22,377	8,41	**22**	-1,425	1,127	-1,175	-2,035	-1,396	-1,612	-3,152	-1,748	-1,667	-5,444	-2,148	-2,143
Stathmin, human	17,292	5,76	**23**	-2,772	-1,133	-1,97	-3,356	-1,88	-1,921	-3,24	-1,517	-2,229	-1,835	-1,169	-1,287
Peptidyl-prolyl cis-trans isomerase A (Rotamase, Cyclophilin A), human	17,870	7,82	**24**	-1,702	-1,041	-1,266	1,023	-1,097	-1,128	1,743	1,446	1,247	-1,313	1,083	-1,102
Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A),human	17,757	8,46	**25**	1,536	1,479	1,468	2,59	1,354	1,581	1,517	1,121	1,218	1,844	1,28	1,623
Galectin-1(Lactose-binding lectin 1, S-Lac lectin 1), human	14,575	5,34	**26**	-1,058	1,271	1,003	1,058	-1,154	-1,026	1,498	1,317	1,11	-1,526	-1,11	-1,287
Profilin (validated), human	15,045	8,44	**27**	1,836	1,92	1,382	1,695	1,538	1,076	1,904	1,588	1,244	2,749	1,967	1,744

Proteomic analysis

To study the factors involved in the different cellular processes, we excised spots of interest with sterile pipette tips and digested the protein samples with trypsin. Then, the digested spots were analyzed in delayed-extraction linear positive mode of the Voyager-DE STR MALDI-TOF-MS.

Table 13 shows the list of identified proteins in FTC-133 cells: for each identified protein the name of the protein and the Mr/pI are listed.

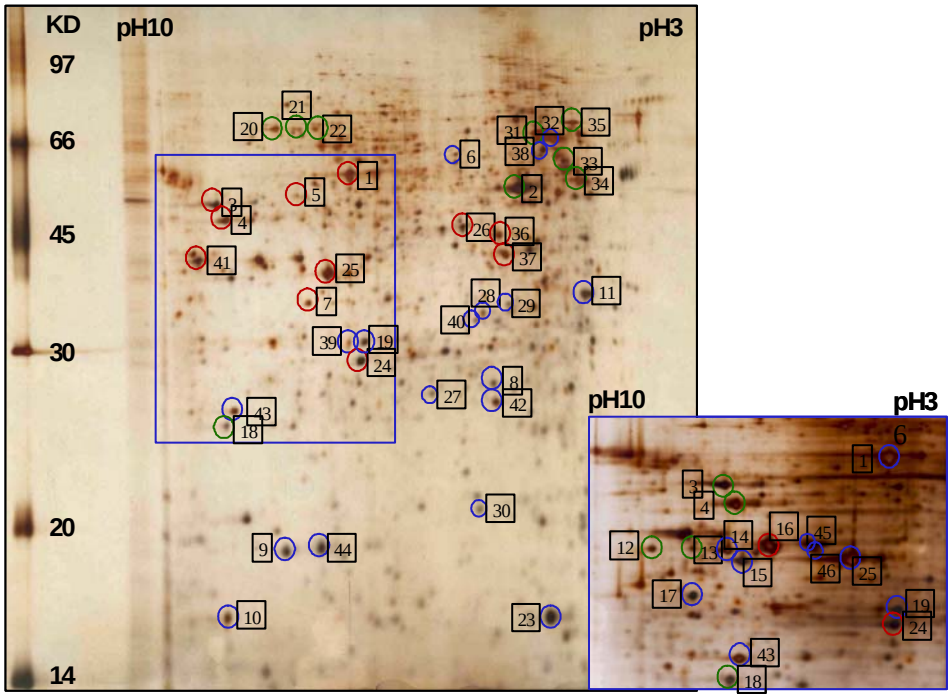
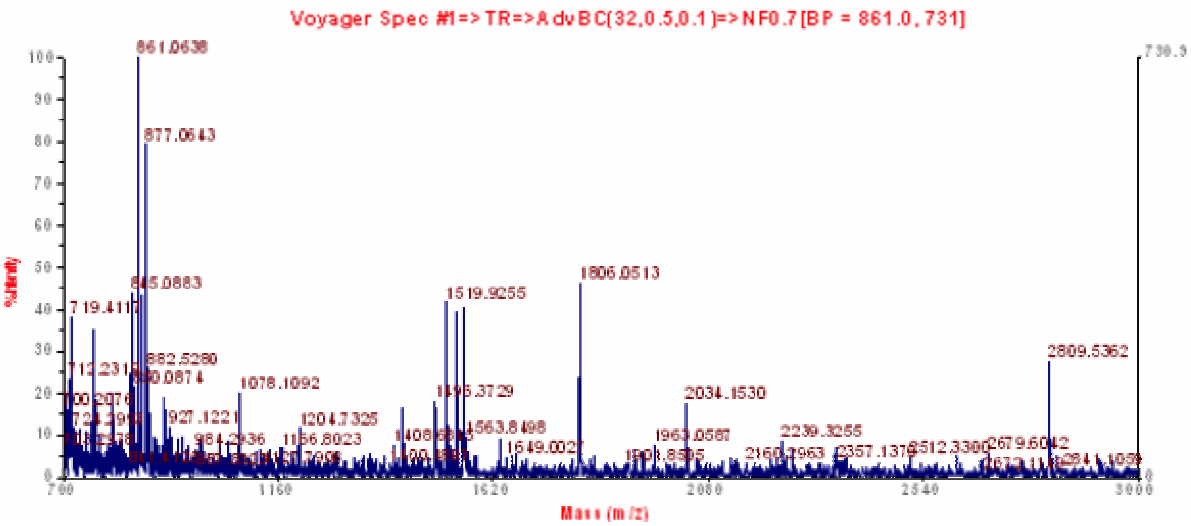


Figure 23: Map of the identified protein spots of FTC-133

a)



b)

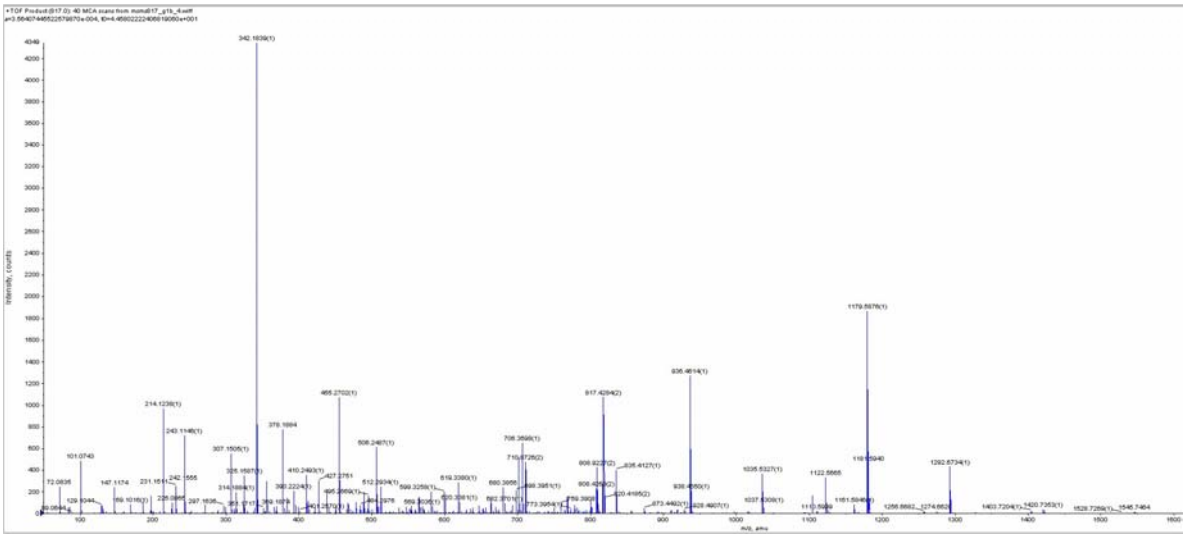


Figure 24: Mass spectrometric analysis of α-enolase spot

a) Spectrum of α-enolase protein with mass 47.008 KDa and PI: 6.99 (Voyager DE PRO MALDI-TOF workstation). b) MS/MS spectrum of α-enolase doubly charged peptide at m/z 817.0 was sequenced as “VNQIGSVTESLQACK” (QSTAR® XL Hybrid LC/MS/MS System).

Table 13: identified protein spots of FTC-133 cells

Spot	Protein	Mr (kDa)	pI
1	Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non- neural enolase),human	47.008	6.99
2	Actin, beta, Homo sapiens	40.978	5.56
3	Phosphoglycerate kinase, human	44.586	8.30
4	Fructose-bisphosphate aldolase A, human	39.264	8.39
5	Citrate synthase, Homo sapiens	51.673	8.13
6	Protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor, human	56.664	6.10
7	Guanine nucleotide-binding protein beta subunit-like protein 12.3,RACK1, human	35.055	7.60
8	Glutathione transferase pi (validated),human	23.341	5.43
9	Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A),human	17.757	8.46
10	Profilin (validated), human	15.045	8.44
11	Tropomyosin, human (Chen)	32.930	4.68
12	hnRNP A2 / hnRNP B1 Splice isoform B1, human	37.407	8.97
13	hnRNP A2 / hnRNP B1 Splice isoform A2, human	35.984	8.67
14	L-lactate dehydrogenase A chain (EC 1.1.1.27, LDH-A), human	36.534	8.46
15	Annexin A2 (Annexin II, Lipocortin II, Calpactin I heavy chain), human	38.449	7.56
16	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), human	35.899	8.58
17	Galectin-3 (Galactose-specific lectin 3, MAC-2 antigen, IgE-binding protein), human	26.041	8.61
18	Transgelin 2, human	22.377	8.41
19	Phosphoglycerate mutase (EC 5.4.2.1) B (validated), human	28.786	6.67
20	Pyruvate kinase, M1 isozyme (Cytosolic thyroid hormon), human	57.769	7.95
21	Pyruvate kinase, muscle splice form M2, human	57.841	7.58
22	Lamin C, human	65.096	6.40
23	Galectin-1(Lactose-binding lectin 1, S-Lac lectin 1), human	14.575	5.34
24	Triosephosphate isomerase (EC 5.3.1.1, TIM), human	26.522	6.51
25	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), human	35.899	8.58
26	Alpha enolase, human	36.286	6.53
27	DJ-1 protein, human	19.834	6.33
28	Proteasome activator PA28 alpha chain, human	28.705	5.78
29	Proteasome activator PA28 beta chain, human	27.331	5.44
30	Stathmin, human	17.292	5.76
31	Tubulin alpha-1 chain (Alpha-tubulin 1), human	50.120	4.94
32	Beta-tubulin (fragment), human	48.848	4.71
33	Vimentin, human	53.522	5.06
34	Vimentin, human	53.522	5.06
35	Protein disulfide isomerase precursor (PDI, Cellular thyroid hormone binding protein, p55), human	57.081	4.76
36	Tubulin alpha-6 chain (Alpha-tubulin 6), human	49.863	4.96
37	Tubulin beta-5 chain, human	49.639	4.78
38	ATP synthase beta chain, mitochondrial precursor (EC 3.6.3.14), human	56.525	5.26
39	Peroxiredoxin 6 (Antioxidant protein 2, 1-Cys peroxiredoxin, 1-Cys PRX), human	24.888	6.02
40	Peroxiredoxin 4 (Prx-IV, Thioredoxin peroxidase AO372), human	30.521	5.86
41	Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37), human	35.509	8.92
42	Peroxiredoxin 2 (Thioredoxin peroxidase 1), human	21.878	5.66
43	Peroxiredoxin 1, human (QSTAR)	22.339	8.18
44	Peptidyl-prolyl cis-trans isomerase A (Rotamase, Cyclophilin A), human	17.870	7.82
45	Annexin II, human	38.580	7.57
46	Fructose-bisphosphate aldolase (EC 4.1.2.13) A (validated), human	39.395	8.30

5 DISCUSSION

5.1 Clinical significances of CD82 and CD63

The progression of thyroid carcinoma from primary tumor to metastasis is a multistep process which includes a loss of intercellular adhesion in primary tumor cells and the entry and circulation of invasive tumor cells in the lymphatic and/or blood vascular compartment. Circulating tumor cells adhere to the surface of the luminal endothelium, gain tissue re-entry by destruction of the underlying subendothelial basement membranes and invade surrounding tissue. Patients with thyroid carcinoma and lymph node and distant metastases have a poor prognosis. Many reports demonstrated that metastasis processes have been correlated with the expression of a wide variety of cellular proteins, including adhesion molecules, growth factors, motility factors, proteases, transcription factors and signalling molecules. However, their precise role in metastasis is largely unknown.

Inactivation of metastasis suppressor genes is one of the most important events contributing to the formation of tumor metastasis. Several tetraspanning TM4SF membrane proteins, including CD9, CD63, CD81, CD82 and CD151, may play a role in cell migration, proliferation and tumor cell metastasis. CD63 expression in melanomas is inversely associated with tumor metastasis [87], while CD82 is considered to be a metastasis suppressor glycoprotein in prostate cancer [25] and non-small-cell lung carcinoma [1]. Moreover, CD82 expression has been demonstrated to inversely correlate with the metastasis potential and the prognosis of several epithelial neoplasms in adults [41, 62, 123, 42, 68, 118]. In pancreatic carcinoma, Guo et al. reported that CD82 mRNA levels were significantly higher in earlier tumor stages (I, II), compared with advanced tumor stages (III, IV) in which lymph node or distant metastases were present. An inverse association between CD82 expression and tumor pathological grading was observed. Poorly differentiated tumors had significantly higher CD82 mRNA levels than those that were moderately or well differentiated. It was shown an inversely association between CD82 expression and tumor pathological grading. In human bladder carcinoma, Yu et al. reported that CD82 was expressed high in all investigated normal bladder tissues. Inflammatory bladder and noninvasive papillary transitional cell carcinomas (TCCs) with low grade (1, 2) also showed a high level of CD82 expression, in contrast to high grade papillary TCCs or invasive TCCs with a low frequency of high expression level of CD82. These data demonstrated that CD82 expression correlated with tumor grading. This result was validated by Ow et al. in a clinical study with a comparatively larger number of patient specimens. In advanced breast cancer, data from Yang et al. showed a significant lower expression of CD82 [123]. They also investigated CD82 in different breast cell lines and found that CD82 expression levels were higher in an immortal, normal-like breast epithelial cell line and nonmetastatic breast cancer

cell lines but substantially lower in highly metastatic breast cancer cell lines. Hashida et al. investigated the relationships between integrin α3 and two tetraspanins in human colon carcinoma [42]. Their data showed a correlation between the expression of tetraspanins members CD9 and CD82 with integrin α3 suggesting that the lack of integrin α3 expression may serve as a suitable indicator for micrometastasis in node-negative patients and can be considered a prognostic factor; they also postulated the change of microdomain complex composed of integrin α3 and CD9 and CD82 may play an important role in tumor progression and metastasis. High frequencies of CD82 down-regulation were found in metastatic oral squamous cell carcinomas (OSCCs) of primary OSCCs and in precancerous lesions and this dowregulation associated with lymph node metastases [118]. Ito et al. were the first to investigate the expression of CD82 in human papillary thyroid carcinoma [54]. Their data indicated that down-regulation of CD82 is significantly related to the progression of papillary carcinoma, including lymph node metastasis, and its anaplastic transformation. The expression of CD82 and several cell proliferation (ki-67, cyclin D1, p27 and pRb) and apoptotic (ssDNA and bcl-2) markers in 19 microcarcinoma patients with clinically apparent metastasis, 14 patients with occult metastasis, and 22 patients without metastasis showed increased cyclin D1 expression together with decreased p27 expression and higher levels of pRb and Ki-67 expression. Furthermore, ssDNA expression was higher and bcl-2 expression was lower in these cases, while CD82 expression was significantly reduced. There was no significant difference in the expression of these proteins between cases demonstrating no and occult metastases. These data demonstrated that significantly higher growth of metastatic tumor is based on cell proliferating activity, apoptosis, and expression of metastatic suppressor. Our data in this study indicated that CD82 expression was down-regulated in metastatic thyroid carcinoma, CD82 expression were lower in advanced tumor stages and this dowregulation associated with lymph node and distant metastases.

We speculated originally that CD82 or CD63 may affect the steps thyroid carcinoma metastasis. In order to validate this hypothesis, the expression of these two members of TM4SF in human thyroid tissue has investigated in the present study to determine the role of both TM4SF members in the progression of thyroid carcinoma formation and metastasis. The expression of CD82 and CD63 in 75 primary thyroid carcinoma and 15 benign nodular goiter post-surgical specimens have been investigated and compared to the clinical pathological parameters, e.g., gender, age, tumor size, regional lymph nodes metastasis, distant metastasis, tumor stage, tumor pathological suptype. Our results show that CD82 was expressed weak in primary thyroid carcinomas in comparison to benign goiter tissue. A further down-regulation or absence of CD82 was observed in metastasized thyroid carcinoma cells which were paralleled by a redistribution of immunoreactive CD82 from the membrane to the cytoplasm in the thyroid carcinoma cells. Expression of CD82 mRNA and protein correlated inversely with the degree of metastasis in thyroid carcinoma and the tumor

stage progression. Thyroid carcinoma joins a growing list of cancers in which the down-regulation of CD82 is associated with tumor progression, including prostate, breast, oesophageal, non-small cell lung, gastric, pancreas, colon and bladder cancer. Suppression of thyroid carcinoma cell motility and invasiveness could be an important function of CD82. Impairment of the migratory behaviour of thyroid carcinoma cells resulting in reduced metastatic potential could result from a decrease in numerous cell properties, including impaired motility, cell adhesiveness, decreased clonal selection and proliferation and increased apoptosis. By contrast, irrespective of the human normal and neoplastic lesions of thyroid tissues investigated the constitutive expression of CD63 suggested a cellular role for this TM4SF member independent of thyroid carcinogenesis. A further investigation of the exact biological function of CD82 in human thyroid carcinoma became distinctly interested for our study.

The mechanism of CD82-mediated inhibition of cancer invasion and metastasis remains unclear. Cell adhesion and cell migration play important roles in variety of physiological and pathological processes such as embryonic development, cancer metastasis, blood vessel formation and remodeling, and inflammation. Because of the specific structure of tetraspanins [122], the extracellular domains can mediate specific protein–protein interactions with laterally associated proteins and unknown ligands, whereas the cytoplasmic regions provide links to cytoskeletal and signaling molecules. Tetraspanins have been implicated in a multitude of biological processes, including fertilization of oocytes, susceptibility to infection by mammalian and plant parasites, metastasis of cancer cells, and cell–cell interactions in the central nervous system (CNS) and the immune system [14, 44]. These pleiotropic involvements are the result of the distinct interactions of individual tetraspanins with specific associated proteins in a molecular network that is known as the tetraspanin web. Tetraspanins and their associated proteins modulate intercellular immune interactions, including adhesion, migration and synapse formation, as well as assist in intracellular interactions as organizers of membrane-signalling complexes [14]. They are also involved in intracellular protein transport, endocytosis and exocytosis, and they function as chaperones or stabilizers of lineage-specific molecules [44]. Although members of the tetraspanin family have common functions in the tetraspanin web, individual members have specific and unique roles that cannot be carried out by other tetraspanins. As a type III integral membrane-anchored protein, CD82 was shown to interact with integrins and human lymphocyte antigens and can engage in aggregation with neighbouring CD82 molecules. While this may implicate CD82 to be involved in cellular adhesion and motility, its potential role of this cell surface glycoprotein in signal transduction or cell growth and differentiation is currently unknown.

For understanding of the potential role of CD82 as metastasis suppressor, we established a CD82 overexpressing thyroid carcinoma cell model, by using a plasmid vector. The stable

transfection was successful; a significant higher CD82 expression was confirmed in transcript and protein levels after the long term antibiotic selection. Based on the preliminary data, we performed MTT test and modified Boyden chamber assay to evaluate the cell proliferation and cell motility between wild type and CD82 overexpressing cells.

Cell viability can be defined as the number of healthy cells in a sample. In MTT test, cell viability is measured by the ability of cells with uncompromised membrane integrity to exclude the dye. This assay is based on the ability of mitochondria in live cells to oxidize thiazolyl blue, a tetrazolium salt, and an insoluble blue formazan product. Since the generation of the blue product is proportional to the mitochondrial dehydrogenase activity, the decrease in the absorbance at 595nm provided a direct measurement of cell death. Our data suggested that the cell proliferation rate of CD82 transfectant was significantly lower than the wild type cells. The decreased cell viability induced by overexpressed CD82 in transfected cells may have caused the reduced cell colony formation. The rate of cell proliferation within any population of cells depends on three parameters: 1) the rate of cell division, 2) the fraction of cells within the population undergoing cell division (growth fraction), and 3) the rate of cell loss from the population due to terminal differentiation or cell death.

Cancer cell migration occurs in a process termed metastasis [92]. Malignant cells must traverse basement membranes during their migration to sites distant from the primary tumor. Metastasis is the migration of cancer cells from their primary tumor formation site to secondary sites where they then invade normal tissue and begin to proliferate and form new tumors. Cell migration is achieved by intracellular and extracellular processes that work together to move a cell from one place to another. The cell migration sequence is composed of four main stages: extension, adhesion, traction and tail retraction. Cell movement begins with the extension of one or more lamellipodia from the leading edge of the cell. Some lamellipodia adhere to the substratum via focal adhesions. Then the bulk of the cytoplasm in the cell body flows forward. The trailing edge of the cell remains attached to the substratum untill the tail eventually detaches and retracts into the cell body. In summary, the control of cellular adhesion and motility is a crucial mechanism responsible for tumor initiation and progression. The genes involved are contributing to malignancy along with genes responsible for cell proliferation and survival.

Quantitative assessment of tumor cell transmigration across artificial anatomic barriers *in vitro* is useful in the study of tumor cell invasion. It allows one to compare the invasiveness of tumor cells and to evaluate the effects of various factors influencing such a process. The modified Boyden chamber system for assessing tumor cell transmigration across endothelial monolayer *in vitro* was used in this study. Employing this modified assay, we compared the abilities of motility of FTC-133 wild type and FTC-133-CD82 transfectans cells. The transmigratory activity was quantified by direct visualization and image analysis (as shown in Figure 18). Our finding suggested that overexpression of CD82 causes impaired motility of

FTC-133 cells. CD82 may decrease the invasiveness of FTC-133 through its adhesion ability on the cell membrane. CD82 may directly or indirectly initiates signals that result in diminished cell motility.

5.2 Tetraspanin-mediated cellular signaling

The function of CD82 is not well understood and it has been proposed that the association of CD82 with other cell-surface proteins may be pivotal in directing its biological activities. CD82 was originally identified as either a membrane protein that induces the intracellular calcium mobilization in lymphocytes, an accessory molecule in T-cell activation, or as the target of a monoclonal antibody that inhibits human T-cell leukemia virus-induced syncytium formation [37, 36, 82]. Studies have indicated that CD82 regulates cell aggregation [103, 114, 113, 58], cell motility [114, 113, 85, 84, 124, 133, 132]. These studies suggest that CD82 is directly or indirectly involved in the outside-in cellular signal transduction modulating cellular behaviour. CD82 and the other members of the tetraspanin family are characterized by their ability to form large multimeric complexes that comprise a wide range of membrane and cytosolic proteins. Such properties have led to the hypothesis that CD82 has a general role in organizing protein complexes, enabling the formation and targeting of specific membrane complexes and thus acts as a “molecular organizer”.

One putative mechanism by which CD82 regulates cellular signal transduction is the direct initiation of signalling events resulting in the attenuation or amplification of other cellular signal transduction events. The biochemical structure of tetraspanins, however, implies that CD82 is less likely to function as an independent signal initiator. It has been reported that integrins associates with CD82 in leukaemia cells, rhabdomyosarcoma cells, hamster ovary cells, and colon carcinoma cells [103, 85, 55, 69, 10, 65, 104]. Integrins are a widely expressed family of heterodimers comprised of a common β chain non-covalently associated with a variety of α chain that confer ligand specificity. In a similar manner as other cell adhesion molecules, integrins are expressed on the cell surface in different states of activation. This fact implies that expression of particular adhesion molecules does not necessarily ensure that the cell adheres to its ligand. Indeed, a number of signaling events initiated by an extracellular signal may culminate in changes in the activation state of integrins and enhance their binding affinity which is mediated by conformational changes in the integrin structure. Following integrin activation, subsequent ligation can lead to changes in gene expression and, consequently, affect cellular growth and differentiation. Accordingly, the function of integrins is not restricted to cell adhesion but extends to signal transduction mediating gene expression and cell growth. CD82 engagement in Jurkat T cells has been shown to trigger important alterations in cell morphology and adhesion properties as well as the association of CD82 with the cytoskeleton [64]. Furthermore, the co-engagement of

CD82 with CD3 in this cell line was accompanied by cytoskeletal changes that were dependent on RHO GTPase activity [22]. The RHO family of small GTPbinding proteins is important in regulating cytoskeletal and integrin-mediated cell adhesion [97].

Our finding suggests that the significant stronger expression of integrin a3 were upregulated by the overexpressing of endogenous CD82 in FTC-133. Therefore, CD82 is also likely to regulate integrin-mediated signalling in human malignant thyroid cells. Although the CD82-integrin complex is prevalent, and the role of CD82 in integrin mediated cell migration is well documented, it still remains to be assessed whether (a) CD82 directly regulates the functional status of its associated integrins, and (b) CD82 indeed affects integrin activity, what regulatory mechanism exists. Microarray data indicated CD82 can also regulate other tetraspanins (e.g. CD9), which correlated closely with tumor cell malignant behaviour and these data are well documented in other experimental and clinical studies.

Recent studies revealed that CD82 attenuates epidermal growth factor (EGF) signaling by accelerating EGF receptor endocytosis through a physical interaction with EGF receptor and also by inhibiting the ligand-induced dimerization of the EGF receptor. Changes in the relative expression of CD82 and EGFR in epithelial tumors (e.g. downregulation of CD82 or overexpression of EGFR) would affect an important suppressive control on EGF-induced signaling. Direct interaction between CD82 and the EGF receptor attenuates EGF receptor-induced lamellipodia formation and migration signaling by regulating EGF receptor dimerization and compartmentalization. According to the hypothesis proposed by He et al., CD82 regulates the functionality of other transmembrane proteins by using a ‘touch and down’ mechanism [11], whereby the protein contacts and subsequently inhibits other proteins. The evidence for the ‘touch and down’ hypothesis includes the existence of an endosome–lysosome-targeting motif on the C-terminal cytoplasmic domain of CD82 [43] and the presence of CD82 in internalization vesicles of B cells [30].

CD82 glycosylation affects tumor cell motility and invasiveness. CD82 has three N-glycosylation sites, they are highly N-glycosylated, Asn157 and Asn198 [84], and function as facilitators of various membrane receptors. Many recent studies indicate that aberrant glycosylation is a result of initial oncogenic transformation, as well as a key event in induction of invasion and metastasis. The integrins bind to distinct, although partially overlapping, subsets of ECM proteins and transmit both mechanical and chemical signals. In addition to imparting polarity to the cell and organizing and remodeling its cytoskeleton during adhesion and migration, these signals exert a stringent control on cell survival and cell proliferation. Most integrins activate Focal Adhesion Kinase (FAK) and thereby Src Family Kinases (SFKs), causing phosphorylation of, and hence signaling from, p130-CAS and paxillin. CD82 attenuates integrin signaling such as FAK-Src-p130CAS-Crk and Rho small GTPases signaling. The CD82-containing tetraspanin web also recruits activated PKC to β1 integrins and regulates the phosphorylation of laminin-binding integrins. As the consequence, cellular

protrusion, possibly cellular retraction, and actin cytoskeleton reorganization are inhibited, and cell migration and cancer invasion are suppressed.

Rho GTPases of the Rho family participate in many physiological processes, such as cell motility, adhesion, cytokinesis, proliferation, differentiation and apoptosis [112]. Each of the Rho proteins acts as a molecular switch, which might turn on or off a coordinately regulated set of intracellular signalling pathways. The switch between the active state (bound to GTP) and inactive state (bound to GDP) is controlled by several types of regulatory factors. Src has been localized to the adherens junction (zonula adherens or ZA) of the apical junctional complex of epithelial cells [116]. Furthermore, Src has been found to induce the tyrosine phosphorylation of ß-catenin and p120 [39], two members of the ZA. Therefore, the Src-family kinases are candidates that warrant investigation. Data by Bokeun Jee et al. suggest that Src mediates the intracellular signaling pathway of CD82 for the induction of homotypic adhesion of human prostate cancer cells [58]. Intercellular and cell–extracellular matrix (ECM) interactions are of great significance in many biological processes of growth, apoptosis, differentiation and cell migration, as well as cancer cell invasion and dissemination. These functions are mediated by many cell adhesion molecules and cell surface receptors. Several families of adhesion molecules have been identified and their synthesis and expression on the cell membrane studied in relation to the invasive and metastatic phenotype by the organ-specific expression pattern of some adhesion molecules [129]. The protein kinase C (PKC) pathways consist of a family of phospholipids-dependent protein kinases. PKC is regulated by a large variety of metabolic pathways involving phospholipids and calcium levels within a cell. The main regulator of the pathway is diacylglycerol (DAG) which appears to recruit PKC to the plasma membrane and cause its activation. The activity of DAG is mimicked by the phorbol-ester tumor promoters. Once activated, PKC can phosphorylate a wide variety of cellular substrates that regulate cell proliferation and differentiation. Responses to PKC appear to vary with the types of PKCs expressed and the types of substrates available within a cell. CD82 can also play a potential role in intracellular-signalling transduction, the biochemical crosslinking of PKC-Tetraspanins indicate a direct link between tetraspanins and intracellularsignalling molecules, which is probably mediated by the tetraspanin cytoplasmic domain [130]. Conventional PKC isoforms participate in the "inside-out" activation of cell adhesion mediated by different integrins [101, 28, 105]. PKC not only appears in focal adhesion complexes [57], but also is required for cell spreading [120]. In addition, PKCα may associate with $ß_1$ integrin and regulate $ß_1$ integrins trafficing [80]. A subset of integrins ($α_3ß_1$, $α_6$ integrins) becomes phosphorylated in a PKC-dependent manner [102, 48, 131, 26]. Phosphorylation of the $α_3$ integrin may regulate cell signaling, morphology, migration, and cytoskeletal organization [122]. Integrins already constitutively associated with TM4SF proteins than become linked to PKC. A novel type of signaling complex “PKC-TM4SF-integrin complex sturcture” was speculated by Zhang XA et

al. [130]. Integrin α_3 and α_6 tails are phosphorylated in a PKC-dependent manner. Our finding suggested that CD82 overexpression upregulated the endogeous integrinα_3 mRNA expressions in FTC-133. The presence of TM4SF linker proteins helps to explain how the association of intracellular PKC may be determined by integrin extracellular domains.

Additionally, all-*trans* retinoic acid (RA) which is a well characterized agent with differentiation-inducing properties is able to induce intercellular adhesion molecule-1 [8], alkaline phosphatase, and type 1 iodothyronine-5'-deiodinase in thyroid carcinoma cells [96]. The redifferentiating effects of RA are confined to at least partly differentiated thyroid cancers and are not seen in anaplastic thyroid cancer [94]. *In vitro* experiments have shown that RA can downregulate sodium iodide symporter (NIS) mRNA and iodide uptake in normal, nontransformed thyrocytes of rats, but upregulate NIS mRNA in the human follicular thyroid carcinoma cell line FTC-133 [107]. In some patients with nonaccumulating thyroid tumors or metastases, RA induces iodide uptake [95]. RA could be useful for a differentiation therapy protocol in selected thyroid carcinoma not responding to L-thyroxine therapy or lacking radioiodide uptake [47]. Our data shown that CD82 mRNA expression in FTC-133 and B-CPAP cells was enhanced by RA stimulation. These results could imply that CD82 can affect indirectly thyroid cell differentiation. It is speculated that CD82 may serve as an indicator for the successful RA treatment in thyroid carcinoma.

The mRNA expression of several genes was evaluated by using RT-PCR (Figure 14). MRP-1/CD9, also named Motility-related protein-1, has been reported to be associated with the biological behavior of solid tumors, especially with their metastatic potential [51, 76]. MRP-1/CD9 is recognized by the murine MAb M31–15 which inhibites cell motility [75]. Cell motility was suppressed in the MRP-1/CD9-expressing human lung cancer cells. In addition, MRP-1/CD9 protein expression was associated with metastasis and a poor prognosis in breast cancer patients [77]. The reduced MRP-1/CD9 gene expression was also correlated with a poor prognosis in non-small cell lung cancer patients [45]. Study by Huang et al. on the relationship between MRP-1/CD9 and cytoskeleton suggested CD9 to regulate the actin cytoskeleton by downregulating the WAVE2 (Wiskott-Aldrich syndrome protein family verprolin-homologous protein 2 protein) through a Wnt-independent signalling pathway [49]. Several tetraspanins bind directly to a few molecular partners to form primary complexes, which might assemble through tetraspanin–tetraspanin interactions to form a network of molecular interactions, the so called tetraspanin web. Adding to these observations, our results indicate that the upregulation of CD82 may contribute to enhanced cell-cell adhesion, suggesting that detachment of tumor cells from the primary lesion might be a step at which CD82 would play a suppressive role during the metastatic cascade.

S100 genes include at least 13 members which are located as a cluster on chromosome 1q21. This protein may have a tumor suppressor function. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number

of cellular processes such as cell cycle progression and differentiation. The S100 proteins are structurally related and share a high degree of homology, particularly in the regions that code for the 2EF-hand motifs which create the Ca^{2+}-binding domains. Wang et al. reported that S100A2 is able to check the hepatocellular carcinoma cell cycle and stop the cell growth and proliferation either *in vitro* or *in vivo* [121]. It shows an opposition function compared to other members in this family. Despite high amino acid sequence homology, S100 members exhibit different expression patterns and functions in human tissues. For instance, S100A4 is defined as a metastasis-associated protein; its expression is associated with adverse clinical outcome. The S100A4 protein belongs to the S100 family of vertebrate-specific proteins possessing both intra- and extracellular functions. Therefore, we checked also the endogenous expression of S100A4. CD82 reduced endogeous S100A4 expression at the transcript level. S100A4 has multiple roles which are reflected in the numerous protein interactions and biological functions suggested for this protein. Whether the S100 family members and tetraspanins interact is not known.

VEGF initiates embryonic vasculogenesis and triggers angiogenic sprouting via its activation of VEGFR-2 on vascular endothelial cells. VEGF activation of VEGFR-1 may limit cell proliferation induced by activation of VEGFR-2 and, may limit VEGF-induced angiogenesis. Vascular endothelial growth factor C (VEGF-C) is one of the most potent directly acting lymphangiogenic factors belonging to the VEGF family. In human PTC lesions VEGF-C immunoreactive protein is overexpressed which correlates with lymph node metastases [127]. Our data demonstrated that overexpressed CD82 reduced VEGF- C expression, thus, potentially Inhibiting the infiltration and angiogenesis. This way, CD82 could play a role in anti-angiogenesis.

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, especially angiogenesis, metastasis. It was reported that tetraspanins can inhibit the Integrin-mediated cell-ECM adhesions [43]. MMP14 is a proteolytic enzyme involved in degradation of extracellular matrix (ECM) and various surface-associated proteins that control cell growth, differentiation and survival, plays crucial roles in molecular carcinogenesis, tumor cell growth, invasion, and angiogenesis. MMP14 is pivotal in controlling the invasiveness of breast cancer cells. No information about MMP14 expression in thyroid malignant lesions.

In our study, overexpressed CD82 reduced the endogenous MMP14 expression of FTC-133 cells. This result supported that CD82 can regulate the membrane inserted matrix metalloproteinase through its significance of indirect effect on collagen degradation of extracellular matrix and basal membranes. It may indicate that CD82 can play an important role in the balance of MMP activity and ECM degradation.

5.3 Proteomic investigation

To determine how CD82 interacts with other cellular molecules as well as their possible modifications we compared the protein spots of wild type FTC-133 to the spots of FTC-133-mock and FTC-133-CD82 transfectans to find the difference of protein production level. Based on these data, the proteomic spectra were obtained from follecular thyroid carcinoma cells FTC-133 using the MALDI-TOF Mass Spectrometry technique (matrix assisted laser desorption/ ionisation time of flight) coupled with hybrid quadrupole time-of-flight mass spectrometry (QTOF-MS).

In the present study we have been able to identifiy over 40 spots in 2-DE corresponding to the proteins extracted from wild type FTC-133, FTC-133-mock and FTC-133-CD82 transfectans (Table 15). According to their biological function, the identified proteins were classified into the following groups:

The most abundant group corresponds to the proteins involved in cytoskeleton (e.g. Lamin C, alpha-1 and Beta-tubulin, Vimentin, Stathmin). The next most abundant group of proteins identified corresponds to those involved in energy production, Glucose metabolisms (e.g. Alpha enolase, Phosphoglycerate kinase, L-lactate dehydrogenase A chain); Ca^{2+} regulation membrane protein (e.g. Annexin II, Transgelin 2); Thioredoxin system (e.g. Peroxiredoxin 1,2,4,6); Apoptosis and cell differentiation (e.g. Galectin-1); Oncogene (e.g. DJ-1 protein).

Our further proteomic investigation will focus on cell membrane and cytoskeletal proteins. It is well known, that microtubules and actin microfilaments are the mean components of networks that mediate nearly all aspects of intracellular transport and cellular movement, playing essential role in secretion, cell motility, mitosis and cell division. Migration of a cell toward an attractant is driven by localized assembly of specialized cytoskeletal proteins. These proteins contribute to directional cell migration. Cancer cell metastasis is fundamentally a cell migration event, and modulation of this activity in cancer cells may generate novel anti-metastatic therapeutical applications. Our proteomic data suggested that CD82 altered these protein productions, e.g. vimentin expression was upregulated by CD82 overexpression in FTC-133-CD82 clones and Tubulin beta-5 chain expression was downregulated in FTC-133-CD82 clones, especially in clone 2. Further proteomic investigations will also focus on the identification and sequencing of important molecules (including e.g. adhesion molecules, growth factors, motility factors, proteases, transcription factors and signalling molecules) as well as their possible transcriptional or translational modifications, which closely associated with tetraspnins, especially with CD82, to clarify and determine the more genuine biological function of this family (TM4SF) in human thyroid malignant neoplasms.

6 CONCLUSION

The present study links the down-regulation of CD82 to progressive thyroid carcinoma metastasis. The diminished CD82 expression in thyroid metastases identifies CD82 as an important target molecule for the study of thyroid carcinoma invasiveness. By contrast, the constitutive expression of CD63 irrespective of the human normal and neoplastic lesions of thyroid tissues investigated, would suggest a cellular role for this TM4SF member independent of thyroid carcinogenesis. Our data suggest that CD82's suppressive action on motility can be extended to human thyroid carcinoma. CD82 served as a malignancy marker for human thyroid carcinoma and has potential diagnostic efficiency and predictive value.

Stable overexpressed CD82 in FTC-133 transfectants were established and demonstrated decreased proliferation and motility compared to FTC-133 and FTC-133-empty plasmid controls (mock). Overexpressed CD82 altered gene expression of FTC-133 and altered genes are closely correlated with cell differentiation, adhesion, apoptosis and migration. CD82 can associate with other tetraspanins to form membrane microdomains that provide a scaffold for the transmission of external stimuli to intracellular-signalling components.

Employing mass spectrometry technology we identified more than 40 proteins of wild type FTC-133 cells and transfectant cells. These proteins involved in cytoskeleton, energy production, Ca^{2+} regulation, thioredoxin system, apoptosis, cell differentiation and Carcinogenesis.

7 REFERENCES

1 Adachi M, Taki T, Ieki Y et al. Correlation of KAI1/CD82 gene expression with good prognosis in patients with non-small cell lung cancer. Cancer Res 56 (1996) 1751-1755

2 Albelda SM, Buck CA. Integrins and other cell adhesion molecules. FASEB J 4 (1990) 2868–2880

3 Aldred MA, Morrison C, Gimm O et al. Peroxisome proliferator-activated receptor gamma is frequently downregulated in a diversity of sporadic nonmedullary thyroid carcinomas. Oncogene 22 (2003) 3412-3416

4 Andersen PE, Kinsella J, Loree TR et al. Differentiated carcinoma of the thyroid with extrathyroidal extension. Am J Surg 170 (1995) 467-470

5 Arihiro K, Inai K. Loss of KAI1/CD82 expression in bone and soft tissue tumors is not associated with lung metastasis. Pathol Res Pract 197 (2001) 627-633

6 Aryee DN, Ambros I, Ambros PF et al. Frequent low level expression in Ewing sarcoma family tumors and widespread absence of the metastasis suppressor KAI1/CD82 in neuroblastoma. Pediatr Res 52 (2002) 279-285

7 Baek SH, Ohgi KA, Rose DW et al. Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. Cell 110 (2002) 55-67

8 Bassi V, Vitale M, Feliciello A et al. Retinoic acid induces intercellular adhesion molecule-1 hyperexpression in human thyroid carcinoma cell lines. J Clin Endocrinol Metab 80 (1995) 1129–1135.

9 Benjamin LE, Golijanin D, Itin A, et al. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103 (1999) 159-165

10 Berditchevski F, Odintsova E. Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. J Cell Biol 146 (1999) 477–492

11 Bienstock RJ, Barrett JC. KAI1, a prostate metastasis suppressor: prediction of solvated structure and interactions with binding partners; integrins, cadherins and cell-surface receptor proteins. Mol Carcinog 32 (2001) 139–153

12 Blood CH, Zetter BR. Tumor interactions with the vasculature: angiogenesis and tumor metastasis. Biochem Biophys Acta 1032 (1990) 89-118

13 Boltze C, Schneider-Stock R, Meyer F et al. Maspin in thyroid cancer: its relationship with p53 and clinical outcome. Oncol Rep 10 (2003) 1783-1787

14 Boucheix C, Rubinstein E. Tetraspanins. Cell Mol Life Sci 58 (2001) 1189–1205

15 Brabant G, Hoang-Vu C, Cetin Y et al. E-cadherin: a differentiation marker in thyroid malignancies. Cancer Res 53 (1993) 4987-4993

16 Brown PD. Matrix metalloproteinase inhibitors in the treatment of cancer. Med Oncol 14 (1997) 1-10

17 Cannon KS, Cresswell P. Quality control of transmembrane domain assembly in the tetraspanin CD82. EMBO J 20 (2001) 2443-2453

18 Cardis E, Kesminiene A, Ivanov V et al.: Risk of thyroid cancer after exposure to 131I in childhood. J Natl Cancer Inst 97 (2005) 724-732

19 Chamber AF, Matrisian LM. Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 89 (1997) 1260-1270

20 Chen JX, Chen Y, DeBusk L et al. Dual functional roles of Tie-2/angiopoietin in TNF-alpha-mediated angiogenesis. Am J Physiol Heart Circ Physiol 287 (2004) 187-195

21 Coburn MC, Wanebo HJ. Prognostic factors and management considerations in patients with cervical metastases of thyroid cancer. Am J Surg 164 (1992) 671-676

22 Delaguillaumie A, Lagaudriere-Gesbert C, Popoff MR et al. Rho GTPases link cytoskeletal rearrangements and activation processes induced via the tetraspanin CD82 in T lymphocytes. J Cell Sci 115 (2002) 433–443

23 Dong JT, Isaacs WB, Barrett JC et al. Genomic organization of the human KAI1 metastasis-suppressor gene. Genomics 41 (1997) 25-32

24 Dong JT, Lamb PW, Rinker-Schaeffer CW et al. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science 268 (1995) 884-886

25 Dong JT, Suzuki H, Pin SS et al. Down-regulation of the KAI1 metastasis suppressor gene during progression of human prostatic cancer infrequently involves gene mutation or allelic loss. Cancer Res 56 (1996) 4387-4390

26 Dumont JA, Bitonti AJ.Modulation of human melanoma cell metastasis and adhesion may involve integrin phosphorylation mediated through protein kinase C. Biochem Biophys Res Commun 204 (1994) 264-272

27 Duren M, Siperstein AE, Shen W et al. Value of stimulated serum thyroglobulin levels for detecting persistent or recurrent differentiated thyroid cancer in high- and low-risk patients. Surgery 126 (1999) 13-19

28 Dustin ML, Springer TA. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature 341 (1989) 619-624

29 Erdmann SM, Heussen N, Moll-Slodowy S et al. CD63 expression on basophils as a tool for the diagnosis of pollen-associated food allergy: sensitivity and specificity. Clin Exp Allergy 33 (2003) 607-614

30 Escola JM, Kleijmeer MJ, Stoorvogel W et al. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J Biol Chem 273 (1998) 20121–20127

31 Escribano L, Orfao A, Diaz Agustin B et al. Human bone marrow mast cells from indolent systemic mast cell disease constitutively express increased amounts of the CD63 protein on their surface. Cytometry 34 (1998) 223-228

32 Fernandez-Real JM, Villabona C, Fernandez-Castaner M et al. Expression of ICAM-1 in distant metastatic thyroid carcinoma. J Endocrinol Invest 19 (1996) 183-185

33 Figge J, del Rosario AD, Gerasimov G et al. Preferential expression of the cell adhesion molecule CD44 in papillary thyroid carcinoma. Exp Mol Pathol 61(1994) 203-211

34 Folkman J. Clinical applications of research on angiogenesis. N Engl J Med 333 (1996) 1757–1763

35 Fukai K, Oh J, Frenk E et al. Linkage disequilibrium mapping of the gene for Hermansky-Pudlak syndrome to chromosome 10q23.1-q23.3. Hum Mol Genet 4 (1995) 1665-1669

36 Fukudome K, Furuse M, Imai T et al. Identification of membrane antigen C33 recognized by monoclonal antibodies inhibitory to human T-cell leukemia virus type 1 (HTLV-1)-induced syncytium formation: altered glycosylation of C33 antigen in HTLV-1-positive T cells. J Virol 66 (1992) 1394-1401

37 Gil ML, Vita N, Lebel-Binay S et al. A member of the tetra spans transmembrane protein superfamily is recognized by a monoclonal antibody raised against an HLA class I-deficient, lymphokine-activated killer-susceptible, B lymphocyte line. Cloning and preliminary functional studies. J Immunol 148 (1992) 2826–2833

38 Godballe C, Asschenfeldt P, Jørgensen KE et al. Prognostic factors in papillary and follicular thyroid carcinomas: p53 expression is a significant indicator of prognosis. Laryngoscope 108 (1998) 243-249

39 Gomez S, del Mont Llosas M, Verdu J et al. Independent regulation of adherens and tight junctions by tyrosine phosphorylation in Caco-2 cells. Biochimica Biophysica Acta 1452 (1999) 121-132

40 Grant CS, Hay ID, Gough IR et al. Local recurrence in papillary thyroid carcinoma: is extent of surgical resection important? Surgery 104 (1988) 954-962

41 Guo X, Friess H, Graber HU et al. KAI1 expression is up-regulated in early pancreatic cancer and decreased in the presence of metastasis. Cancer Res 56 (1996) 4876-4880

42 Hashida H, Takabayashi A, Tokuhara T et al. Integrin alpha3 expression as a prognostic factor in colon cancer: association with MRP-1/CD9 and KAI1/CD82. Int J Cancer 97 (2002) 518-525

43 He B, Liu L, Cook GA et al. Tetraspanin CD82 attenuates cellular morphogenesis through down-regulating integrin a6-mediated cell adhesion. J Biol Chem 280 (2005) 3346–3354

44 Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. Annu Rev Cell Dev Biol 19 (2003) 397–422

45 Higashiyama M, Taki T, Ieki Y et al. Reduced motility related protein-1 (MRP-1/CD9) gene expression as a factor of poor prognosis in non-small cell lung cancer. Cancer Res 55 (1995) 6040-6044

46 Hoang-Vu C, Boltze C, Gimm O et al. Expression of telomerase genes in thyroid carcinoma. Int J Oncol 21 (2002) 265-272

47 Hoang-Vu C, Dralle H, Scheumann G et al. Gene expression of differentiation- and dedifferentiation markers in normal and malignant human thyroid tissues. Exp Clin Endocrinol 100 (1992) 51-56

48 Hogervorst F, Kuikman I, Noteboom E et al. The role of phosphorylation in activation of the alpha 6A beta 1 laminin receptor. J Biol Chem 268 (1993) 18427-18430

49 Huang CL, Ueno M, Liu D et al. MRP-1/CD9 gene transduction regulates the actin cytoskeleton through the downregulation of WAVE2. Oncogene (2006)

50 Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69 (1992) 11–25

51 Ikeyama S, Koyama M, Yamaoko M et al. Suppression of cell motility and metastasis by transfection with human motility-related protein (MRP-1/CD9) DNA. J Exp Med 177 (1993) 1231-1237

52 Iribarren C, Haselkorn T, Tekawa IS et al. Cohort study of thyroid cancer in a San Francisco Bay area population. Int J Cancer 93 (2001) 745-750

53 Ito Y, Uruno T, Takamura Y et al. Papillary microcarcinomas of the thyroid with preoperatively detectable lymph node metastasis show significantly higher aggressive characteristics on immunohistochemical examination. Oncology 68 (2005) 87-96

54 Ito Y, Yoshida H, Uruno T et al. KAI1 expression in thyroid neoplasms: its linkage with clinicopathologic features in papillary carcinoma. Pathol Res Pract 199 (2003) 79-83

55 Iwata S, Kobayashi H, Miyake-Nishijima R et al. Distinctive signaling pathways through CD82 and beta1 integrins in human T cells. Eur J Immunol 32 (2002) 1328–1337

56 Jackson P, Kingsley EA, Russell PJ. Inverse correlation between KAI1 mRNA levels and invasive behaviour in bladder cancer cell lines. Cancer Lett 156 (2000) 9-17

57 Jaken S, Leach K, Klauck T. Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. J Cell Biol 109 (1989) 697-704

58 Jee B, Jin K, Hahn JH et al. Metastasis-suppressor KAI1/CD82 induces homotypic aggregation of human prostate cancer cells through Src-dependent pathway. Exp Mol Med 35 (2003) 30–37

59 Kebebew E, Peng M, Reiff E et al. ECM1 and TMPRSS4 are diagnostic markers of malignant thyroid neoplasms and improve the accuracy of fine needle aspiration biopsy. Ann Surg 242 (2005) 353-361

60 Kehlen A, Lendeckel U, Dralle H et al. Biological significance of aminopeptidase N/CD13 in thyroid carcinomas. Cancer Res 63 (2003) 8500-8506

61 Kim JH, Kim B, Cai L et al.Transcriptional regulation of a metastasis suppressor gene by Tip60 and beta-catenin complexes. Nature 434 (2005) 921-926

62 Kim Ow, Warick Delprado, Richard Fisher et al. Relationship between expression of the KAI1 metastasis suppressor and other markers of advanced bladder cancer. J of Pathology 191 (2000) 39-47

63 Kondoh M, Ueda M, Ichihashi M et al.Decreased expression of human melanoma-associated antigen ME491 along the progression of melanoma pre-canceroses to invasive and metastatic melanomas. Melanoma Res 3 (1993) 241-245

64 Lagaudriere-Gesbert C, Lebel-Binay S, Hubeau C et al. Signaling through the tetraspanin CD82 triggers its association with the cytoskeleton leading to sustained morphological changes and T cell activation. Eur J Immunol 28 (1998) 4332–4344

65 Lee JH, Seo YW, Park SR et al. Expression of a splice variant of KAI1, a tumor metastasis suppressor gene, influences tumor invasion and progression. Cancer Res 63 (2003) 7247–7255

66 Lennard CM, Patel A, Wilson J et al. Intensity of vascular endothelial growth factor expression is associated with increased risk of recurrence and decreased disease-free survival in papillary thyroid cancer. Surgery 129 (2001) 552-558

67 Liotta LA, Stetler-Stevenson WG. Metalloproteinases and cancer invasion. Semin Cancer Biol 1 (1990) 99-106

68 Lombardi DP, Geradts J, Foley JF et al. Loss of KAI1 expression in the progression of colorectal cancer. Cancer Res 59 (1999) 5724-5731

69 Mannion BA, Berditchevski F, Kraeft SK et al. Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associated with integrin alpha 4 beta 1 (CD49d/CD29). J Immunol 157 (1996) 2039–2047

70 Marreiros A, Dudgeon K, Dao V et al. evidence for a possible mechanism underlying loss of KAI1 expression in cancer cells. Oncogene 24 (2005) 637-649

71 Mashimo T, Bandyopadhyay S, Goodarzi G et al. Activation of the tumor metastasis suppressor gene, KAI1, by etoposide is mediated by p53 and c-Jun genes. Biochem Biophys Res Commun 274 (2000) 370-376

72 Mazzaferri EL, Jhiang SM. Long-term impact of initial surgical and medical therapy on papillary and follicular thyroid cancer. Am J Med 97 (1994) 418-428

73 Mazzaferri EL: Treating differentiated thyroid carcinoma: where do we draw the line? Mayo Clin Proc 66 (1991) 105-111

74 Metzelaar MJ, Wijngaard PL, Peters PJ et al. CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. J Biol Chem 266 (1991) 3239-3245

75 Miyake M, Koyama M, Seno M et al. Identification of the motility-related protein (MRP-1), recognized by monoclonal antibody M31–15, which inhibits cell motility. J Exp Med 174 (1991) 1347-1354

76 Miyake M, Nakano K, Ieki Y et al. Motility related protein 1 (MRP-1/CD9) expression: inverse correlation with metastases in breast cancer. Cancer Res 55 (1995) 4127-4131

77 Miyake M, Nakano K, Itoi S et al. Motility-related protein-1 (MRP-1/CD9) reduction as a factor of poor prognosis in breast cancer. Cancer Res 56 (1996) 1244-1249

78 Miyamoto H, Homma M, Hotta H. Molecular cloning of the murine homologue of CD63/ ME491 and detection of its strong expression in the kidney and activated macrophages. Biochim Biophys Acta 1217 (1994) 312-316

79 Moustafa AS, Nicolson GL. Breast cancer metastasis-associated genes: prognostic significance and therapeutic implications. Oncol Res 9 (1997) 505-525

80 Ng T, Shima D, Squire A et al. PKCalpha regulates beta1 integrin-dependent cell motility through association and control of integrin traffic. EMBO J 18 (1999) 3909-3923.

81 Nishibori M, Cham B, McNicol A et al. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak syndrome, and appears identical to granulophysin. J Clin Invest 91 (1993) 1775-1782

82 Nojima Y, Hirose T, Tachibana K et al. The 4F9 antigen is a member of the tetra spans transmembrane protein family and functions as an accessory molecule in T cell activation and adhesion. Cell Immunol 152 (1993) 249–260

83 Okochi H, Mine T, Nashiro K et al. Expression of tetraspans transmembrane family in the epithelium of the gastrointestinal tract. J Clin Gastroenterol 29 (1999) 63-67

84 Ono M, Handa K, Withers DA et al. Glycosylation effect on membrane domain (GEM) involved in cell adhesion and motility: a preliminary note on functional alpha3, alpha5-CD82 glycosylation complex in ldlD 14 cells. Biochem Biophys Res Commun 279 (2000) 744–750

85 Ono M, Handa K, Withers DA et al. Motility inhibition and apoptosis are induced by metastasis-suppressing gene product CD82 and its analogue CD9, with concurrent glycosylation. Cancer Res 59 (1999) 2335–2339

86 Pacini F, Vorontsova T, Molinaro E et al.: Prevalence of thyroid autoantibodies in children and adolescents from Belarus exposed to the Chernobyl radioactive fallout. Lancet 352 (1998) 763-766

87 Radford KJ, Mallesch J, Hersey P. Suppression of human melanoma cell growth and metastasis by the melanoma-associated antigen CD63 (ME491). Int J Cancer 62 (1995) 631-635

88 Radford KJ, Thorne RF, Hersey P. Regulation of tumor cell motility and migration by CD63 in a human melanoma cell line. J Immunol 158 (1997) 3353-3358

89 Ruiz-Garcia J, Ruiz de Almodóvar JM, Olea N et al. Thyroglobulin level as a predictive factor of tumoral recurrence in differentiated thyroid cancer. J Nucl Med 32 (1991) 395-398

90 Sanders LE, Cady B. Differentiated thyroid cancer: reexamination of risk groups and outcome of treatment. Arch Surg 133 (1998) 419-425

91 Scheumman GF, Hoang-Vu C, Cetin Y et al. Clinical significance of E-cadherin as a prognostic marker in thyroid carcinomas. J Clin Endocrinol Metab 80 (1995) 2168-2172

92 Schiemann WP, Blobe GC, Kalume DE et al. Context-specific effects of fibulin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion. Fibulin-5 is induced by transforming growth factor-beta and affects protein kinase cascades. J Biol Chem 277 (2002) 27367-27377

93 Schmidt NO, Westphal M, Hagel C, et al. Levels of vascular endothelial growth factor, hepatocyte growth factor/scatter factor and basic fibroblast growth factor in human gliomas and their relation to angiogenesis. Int J Cancer 84 (1999)10-18

94 Schmutzler C, Brtko J, Bienert K et al. Effects of retinoids and role of retinoic acid receptors in human thyroid carcinomas and cell line derived therefrom. Exp Clin Endocrinol Diabetes 104 (1996) 16–19

95 Schmutzler C, Winzer R, Meissner-Weigl J et al. Retinoic acid increases sodium/iodide symporter mRNA levels in human thyroid cancer cell lines and suppresses expression of functional symporter in non transformed FRTL-5 rat thyroid cells. Biochem Biophys Res Commun 240 (1997) 832–838

96 Schreck R, Schnieders F, Schmutzler C et al. Retinoids stimulate type I iodothyronine 5'-deiodinase activity in human follicular carcinoma cell lines. J Clin Endocrinol Metab 79 (1994) 791–798

97 Schwartz MA, Shattil SJ.Signaling networks linking integrins and rho family GTPases. Trends Biochem Sci 25 (2000) 388–391

98 Sellers M, Beenken S, Blankenship A et al. Prognostic significance of cervical lymph node metastases in differentiated thyroid cancer. Am J Surg 164 (1992) 578-581

99 Seve P, Isaac S, Tredan O et al. Expression of class III {beta}-tubulin is predictive of patient outcome in patients with non-small cell lung cancer receiving vinorelbine-based chemotherapy. Clin Cancer Res 11 (2005) 5481-5486

100 Shah JP, Loree TR, Dharker D et al. Prognostic factors in differentiated carcinoma of the thyroid gland. Am J Surg 164 (1992) 658-661

101 Shattil SJ, Brass LF. Induction of the fibrinogen receptor on human platelets by intracellular mediators. J Biol Chem 262 (1987) 992-1000

102 Shaw LM, Messier JM, Mercurio AM.The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the alpha 6 beta 1 integrin. J Cell Biol 110 (1990) 2167-2174

103 Shibagaki N, Hanada K, Yamaguchi S et al. Functional analysis of CD82 in the early phase of T cell activation: roles in cell adhesion and signal transduction. Eur J Immunol 28 (1998) 1125–1133

104 Shibagaki N, Hanada K, Yamashita H et al. Overexpression of CD82 on human T cells enhances LFA-1 / ICAM-1-mediated cell-cell adhesion: functional association between CD82 and LFA-1 in T cell activation. Eur J Immunol 29 (1999) 4081–4091

105 Shimizu Y, Van Seventer GA, Horgan KJ et al. Regulated expression and binding of three VLA (beta 1) integrin receptors on T cells. Nature 345 (1990) 250-253

106 Siironen P, Louhimo J, Nordling S et al. Prognostic factors in papillary thyroid cancer: an evaluation of 601 consecutive patients. Tumour Biol 26 (2005) 57-64

107 Simon D, Köhrle J, Schmutzler C et al. Redifferentiation therapy of differentiated thyroid carcinoma with retinoic acid: basics and first clinical results. Exp Clin Endocrinol Diabetes 104 (1996) 13–15

108 Sridhar SC, Miranti CK. Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. Oncogene 25 (2005) 2367-2378

109 Stark AM, Tongers K, Maass N et al. Reduced metastasis-suppressor gene mRNA-expression in breast cancer brain metastases. J Cancer Res Clin Oncol 131 (2005) 191-198

110 Staunton MD. Thyroid cancer: a multivariate analysis on influence of treatment on long-term survival. Eur J Surg Oncol 20 (1994) 613-621

111 Suri C, McCLain J, Thurston G et al. Increased vascularization in mice overexpressing angiopoietin-1. Science 282 (1998) 468-471

112 Symons M, Derry JM, Karlak B et al. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell 84 (1996) 723-734

113 Takaoka A, Hinoda Y, Sato S et al. Reduced invasive and metastatic potentials of KAI1-transfected melanoma cells. Jpn J Cancer Res 89 (1998) 397–404

114 Takaoka A, Hinoda Y, Satoh S et al. Suppression of invasive properties of colon cancer cells by a metastasis suppressor KAI1 gene.Oncogene 16 (1998) 1443–1453

115 Tarbe N, Losch S, Burtscher H et al. Identification of rat pancreatic carcinoma genes associated with lymphogenous metastasis. Anticancer Res 22 (2002) 2015-2027

116 Tsukita S, Oishi K, Akiyama T et al. Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. J Cell Biol 113 (1991) 867-879

117 Tsutsumi S, Shimura T, Morinaga N et al. Loss of KAI1 expression in gastric cancer. Hepatogastroenterology 52 (2005) 281-284

118 Uzawa K, Ono K, Suzuki H et al. High Prevalence of Decreased Expression of KAI1 Metastasis Suppressor in Human Oral Carcinogenesis. Clin Cancer Res 8 (2002) 828–835

119 Voutilainen PE, Multanen MM, Leppäniemi AK et al. Prognosis after lymph node recurrence in papillary thyroid carcinoma depends on age. Thyroid 11 (2001) 953-957

120 Vuori K, Ruoslahti E. Activation of protein kinase C precedes alpha 5 beta 1 integrin-mediated cell spreading on fibronectin. J Biol Chem 268 (1993) 21459-21462

121 Wang X, Chen L, Zeng Y et al. Calcium-binding protein S100A2 in the retardation of growth and proliferation of hepatocellular carcinoma cells. Zhonghua Zhong Liu Za Zhi 23 (2001) 363-365

122 Wright MD, Tomlinson MG. The ins and outs of the transmembrane 4 superfamily. Immunol Today 15 (1994) 588-594

123 Yang X, Wei L, Tang C et al. KAI1 Protein Is Down-Regulated during the Progression of Human Breast Cancer. Clin Cancer Res 6 (2000) 3424-3429

124 Yang X, Wei LL, Tang C et al. Overexpression of KAI1 suppresses *in vitro* invasiveness and *in vivo* metastasis in breast cancer cells. Cancer Res 61 (2001) 5284–5288

125 Yoshida BA, Sokoloff MM, Welch DR et al. Metastasis-Suppressor Genes: a Review and Perspective on an Emerging Field. J Natl Cancer Inst 92 (2000) 1717-1730

126 Yu AE, Hewitt RE, Connor EW et al. Matrix metalloproteinases: novel targets for directed cancer therapy. Drugs Aging 11 (1997) 229-244

127 Yu XM, Lo CY, Chan WF et al. Increased expression of vascular endothelial growth factor C in papillary thyroid carcinoma correlates with cervical lymph node metastase. Clin Cancer Res 11 (2005) 8063-8069

128 Yu Y, Yang JL, Markovic B et al. Loss of KAI1 messenger RNA expression in both high-grade and invasive human bladder cancers. Clin Cancer Res 3 (1997) 1045-1049

129 Zetter BR. Adhesion molecules in tumor metastasis. Semin Cancer Biol 4 (1993) 219–229

130 Zhang XA, Bontrager AL, Hemler ME. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific β1 integrins. J Biol Chem 276 (2001) 25005–25013

131 Zhang XA, Bontrager AL, Stipp CS et al. Phosphorylation of a conserved integrin alpha 3 QPSXXE motif regulates signaling, motility, and cytoskeletal engagement. Mol Biol Cell 12 (2001) 351-365.

132 Zhang XA, He B, Zhou B et al. Requirement of the p130CAS-Crk coupling for metastasis suppressor KAI1/CD82-mediated inhibition of cell migration. J Biol Chem 278 (2003) 27319–27328

133 Zhang XA, Lane WS, Charrin S et al. EWI2/PGRL associates with the metastasis suppressor KAI1/CD82 and inhibits the migration of prostate cancer cells. Cancer Res 63 (2003) 2665–2674

8 THESEN

1. Die Familie der Tetraspanine (TM4SF) umfaßt 26 Mitglieder (darunter CD82 und CD63). Sie ist durch vier Transmembrandomänen, eine kleine und eine größere extrazelluläre Schleife, sowie durch intrazelluläre Domänen mit einem N- und einem C-Terminus charakterisiert.

2. CD82 besitzt eine metastasierungssupprimierende Eigenschaft in humanem Prostatakarzinom. In mehreren humanen solidären Tumoren spielt CD82 eine wichtige Rolle in der Zelldifferenzierung, Apoptose, Zelladhäsion und Tumor Metastasierung. CD63 besitzt die Fähigkeit die Motilität von humanen malignen Melanom Zellen zu regulieren.

3. Mittels semiquantitativer RT-PCR und Immunhistochemie, wurde in allen untersuchten benignen Strumageweben eine hohe Expression von CD82 detektiert. Dagegen zeigten die malignen Schilddrüsengeweben eine stark bis mäßig (66,7% mRNA/ 58% Protein) oder eine schwache bis keine Expression.

4. Es zeigte sich eine signifikant stärkere CD82 Expression in nicht metastasierten Karzinomen (pN0/ M0) im Vergleich zu Lymphogen metastasierten und hämatogenen metastasierten Karzinomen (pN1/ M1).

5. CD63 konnte in allen malignen und benignen Schilddrüsengeweben nachgewiesen werden. In besondere Masse zeigte sich in den FTC Geweben eine höhere CD63 Expression als in benignen Geweben. Die Expressionsmuster korrelierten nicht mit den Tumorstadien.

6. Mittels eines eukaryotischen Expressionsvectors wurde ein stabiles CD82-überexprimiertes Zellmodel aus einer follikulären Schilddrüsenkarzinom Zelllinie (FTC-133) etabliert.

7. Die Resultate der MTT-assays und Migrations-assays zeigen, dass die Überexpression von CD82 eine signifikante Verringerung der Zell-Proliferationsrate und der Zell-Motilität in den FTC-133 Zellen induziert.

8. Veränderungen der Genexpression durch die Überexpression von CD82 wurden mittles RT-PCR analysiert. Dabei wurden die hoch- oder runterregulierten Gene in den stabilen CD82 überexprimirenden Transfektanten im Vergleich zum Wildtyp betrachtet. Hier

konnten Gene gefunden werden, welche mit verschiedenen Zellulären Prozessen wie Zelldifferenzierung, Apoptose, Zelladhäsion sowie Metastasierung assoziiert sind.

9. Mittels Retinsäure, welche für die Induktion der Zelldifferenzierung eingesetzt wird, konnte in den FTC-133 Zellen eine CD82 Expression induziert werden.

10. Proteomanlysen von FTC-133 Zellen wurden mit Hilfe von 2D-Gelelektrophoresen und Massenspektrometrie (MALDI-TOF/ Q-TOF) durchgeführt. Anschließend wurden die identifizierten Proteine nach ihren Funktionen sortiert. Darunter befinden sich Ca^{2+}-regulierende Membranproteine; Proteine welche den Glucosemetabolismus regulieren; Proteine des Thioredoxin-System; Proteine des Zytoskeletts; Apoptose-regulierende Proteine; sowie Proteine der Zelldifferenzierung und Onkogene.

11. Nach diesen Untersuchungen, kann CD82 als ein Malignitäts-Marker der humanen Schilddrüsenkarzinome dienen, dessen Expression mit tumor staging, insbesondere mit Metastasierung korreliert. Jedoch sollte diese Funktion in humanen Schilddrüsenkarzinomen weiterhin untersucht werden.

12. CD63 wird unabhängig vom Tumorstatus und Histologie, ubiquitär in Schilddrüsenkarzinomen exprimiert.

13. Um ein weiteres Verständnis über die Funktion und den Einfluss von CD82 in malignen Tumoren zu erhalten, sollten nächste Untersuchungen im Bereich der Signaltransduktionskaskade der Tetraspanine durchgeführt werden.

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SELBSTÄNDIGKEITSERKLÄRUNG

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Halle/Saale, im September 2006 Zhouxun Chen

PUBLICATIONS AND PRESENTATIONS

Publications

1. **Chen Z,** Mustafa T, Trojanowicz B, Brauckhoff M, Gimm O, Schmutzler C, Köhrle J, Holzhausen HJ, Kehlen A, Klonisch T, Finke R, Dralle H, Hoang-Vu C. CD82 and CD63 in thyroid cancer. Int J Mol Med (2004) 517-527
2. Liu Y, Chen L, Peng SY, **Chen Z**, Hoang-Vu C. Role of CD97 (stalk) and CD55 as molecular markers for prognosis and therapy of gastric carcinoma patients. J Zhejiang Univ Sci B (2005) 913-918
3. Liu Y, Chen L, Peng SY, **Chen Z**, Gimm O, Finke R, Hoang-Vu C. The expression of CD97 (EGF) and its ligand CD55 on marginal epithelium is related to higher stage and depth of tumor invasion of gastric carcinomas. Oncol Rep (2005) 1413-1420
4. **Chen Z**, Trojanowicz B, Finke R, Dralle H, Hoang-Vu C. Expression of an anti-Apoptosis Gene Survivin in human thyroid and small interfering RNAs target thyroid carcinoma. J Surg Oncol (In submission)
5. B Trojanowicz, A Winkler, K Hammje, **Z Chen**, C Sekulla, D Glanz, C Schmutzler, B Mentrup, S Hombach-Klonisch, T Klonisch, R Finke, J Köhrle, H Dralle, C Hoang-Vu. Retinoic acid actions are connected with downregulation of ENO1 gene products in the follicular thyroid carcinoma cell line FTC-133. J Biol Chem (submitted)
6. 柳咏，陈力，彭淑牖，**陈周浔,** Hoang-Vu C. 胃癌组织中 $CD97^{STALK}$ 和 CD55 的表达及其临床病理学意义。实用肿瘤杂志 J Practical Onco (2005) 327-331

Abstracts

1. **Chen Z**, Mustafa T, Dralle H, Hoang-Vu C, Hombach-Klonisch S, Klonisch T. IGF-1, IGF-1Rα and IGFBP3 in human medullary thyroid carcinoma. 19th Annual Meeting of German Society of Anatomy, Würzburg, Germany, 2002; 55-56
2. Mustafa T, **Chen Z**, Erhuma M, Dralle H, Hoang-Vu C, Klonisch T. CD55 expression in medullary thyroid carcinomas. 19th Annual Meeting of German Society of Anatomy, Würzburg, Germany, 2002; 101
3. **Chen Z**. CD82-Expression in Schilddrüsen. 17th Annual Meeting of German experimental Thyroid Research, Institute of Experimental Endocrinology, Charite medicine centre, Berlin, 2002 (Oral presentation)
4. C. Hoang-Vu, **Z. Chen**, T. Mustafa, M. Erhuma, S. Hombach-Klonisch, T.P. Nguyen, M. Brauckhoff, H. Dralle. CD82: a novel metastasis suppressor gene in human thyroid

carcinoma. 40th World Congress of the International Society of Surgery, Bangkok, Thailand, 2003

5. **Chen Z**, Trojanowicz B, Mustafa T, Brauckhoff M, Gimm O, Dralle H, Hoang-Vu C. Hot-thyroidology: Expression of an Anti-Apoptosis Gene Survivin in human thyroid. Annual Meeting European Thyroid Association, Edinburgh, Scotland, UK, 2003 (Oral presentation)
6. **Chen Z**, Winkler A, Hammje K, Mentrup B, Schmutzler C, Köhrle J and Hoang- Vu C. Proteomanalyse mit MALDI-TOF am Beispiel der Zelllinie FTC- 133. 18th Annual Meeting of German experimental Thyroid Research, Surgery centre, Martin-Luther-University Halle-Wittenberg, 2003 (Oral presentation)
7. Trojanowicz B, **Chen Z**, Mustafa T, Dralle H, Finke R, Hoang-Vu C. Expression of two ARE directed proteins –AUF1 and HuR in follicular thyroid carcinoma cell line (FTC 133). Experimental and Clinical Endocrinology & Diabetes, S28. Nr. P24, Supplement 1. Vol. 112, 2004, 48th Annual Meeting of German Society of Endocrinology (DGE) Dresden, March 3rd- 6rd, 2004
8. **Chen Z**, Trojanowicz B, Mustafa T, Zschoyan R, Brauckhoff M, Gimm O, Dralle H, Hoang-Vu C. Effects of CD82-siRNA in thyroid carcinoma cell line FTC-133. Experimental and Clinical Endocrinology & Diabetes, S29. Nr. P30, Supplement 1. Vol. 112, 2004, 48th Annual Meeting of German Society of Endocrinology, Dresden, Germany, 2004
9. **Chen Z**, Trojanowicz B, Gimm O, Schmutzler C, Köhrle J, Kehlen A, Klonisch T, Hombach-Klonisch S, Finke R, Dralle H, Hoang-Vu C. Small Interfering RNAs suppress the expression of endogenous survivin in follicular thyroid Cancer Cells. Journal of Cancer Research and Clinical Oncology, Vol.130, S93_P0437, 26th German Cancer Congress, 2004
10. **Chen Z**, Trojanowicz B, Mustafa T, Zschoyan R, Brauckhoff M, Gimm O, Dralle H, Hoang-Vu C.CD82-siRNAs suppress the expression of CD82 in follicular thyroid carcinoma cell line. American Association for Cancer Research (ARCC) Annual Meeting of cancer research, Orlando, Florida, USA, March 2004
11. Mustafa T, Gimm O, Rothe U, **Chen Z**, Trojanowicz B, Erhuma M, Zschoyan R, Kehlen A, Dralle H, Hoang-Vu C. Betulinic acid is an effective CD97 inhibitor in the human medullary thyroid carcinoma cell line MTC-SK. American Association for Cancer Research (ARCC) Annual Meeting of cancer research, Orlando, Florida, USA, March 2004
12. **Chen Z**, Mustafa T, Erhuma M, Hombach-Klonisch. S, Nguyen T.P., Brauckhoff M, Dralle H, Hoang-Vu C. CD82: a novel metastasis suppressor gene in human thyroid carcinoma. Conference of the international Association of Endocrine Surgeons, June 13-17, Uppsala, Sweden, 2004

13. Winkler, B. Trojanowicz, K. Hammje, **Z. Chen**, R. Finke, H. Dralle and C. Hoang-Vu. Retinoic acid induces post-translational modifications of alpha-enolase in the thyroid cell line FTC-133. Conference of the international Association of Endocrine Surgeons, June 13-17, Uppsala, Sweden, 2004
14. Trojanowicz B, **Chen Z**, Brodauf L, Lüftenegger C, Mustafa T, Finke R, Dralle H and Hoang-Vu C. AUF-1 expression in the thyroid. Conference of the international Association of Endocrine Surgeons, June 13-17, Uppsala, Sweden, 2004
15. Trojanowicz B, Winkler A, Hammje K, **Chen Z**, Sekulla C, Glanz D, Schmutzler C, Mentrup B, Hombach-Klonisch S, Klonisch T, Finke R, Köhrle J, Dralle H, Hoang-Vu C. Effect of RA on proteome of thyroid carcinoma cell line FTC-133. 23th DECHEMA-Annual meeting of Biotechnology and German society for Proteomic Research (DGPF), Bioperspectives, Wiesbaden, Germany, 2005
16. Trojanowicz B,Winkler A, Hammje K, **Chen Z**, Sekulla C, Glanz D, Schmutzler C, Mentrup B, Hombach-Klonisch S, Klonisch T, Finke R, Köhrle J, Dralle H and Hoang-Vu C. Influence of retinoic acid on post-translational modifications in follicular thyroid carcinoma cell line FTC-133. 49th Annual Meeting of German Society of Endocrinology, Münster, Germany, 2005
17. **Chen Z**, Trojanowicz B, Hoang-Vu C, Finke R. Proteome Analyses of Keratinocytes in co-cultured with Fibroblasts. International Symposium for Burn Research, Burn center of Changhai hospital, Shanghai, China, 2005 (Oral presentation)
18. Trojanowicz B, **Chen Z**, Finke R, Dralle H, Hoang-Vu C. Influence of AUF1 targeting siRNA on cell cycle-related proteins in thyroid cancer cell lines. 50th Annual Meeting of German Society of Endocrinology, Essen, Germany, 2006
19. Trojanowicz B, Winkler A, Hammje K, **Chen Z**, Sekulla C. Glanz D, Schmutzler C, Mentrup B, Hombach-Klonisch S, Klonisch T, Finke R, Köhrle J, Dralle H and Hoang-Vu C. Retinoic acid actions are connected with downregulation of ENO1 gene products in the follicular thyroid carcinoma cell line FTC-133. 50th Annual Meeting of German Society of Endocrinology, Essen, Germany, 2006
20. **Chen Z**, Chen L, Hoang-Vu C. Overexpression of CD82 reduced the proliferation and migration of human follicular thyroid carcinoma cells (FTC-133) *in vitro.* 10th Congress of the Asian Association of Endocrine Surgeons (AsAES), 12 - 15 March, Hongkong, China, 2006

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