

**Possible pathological role of galectin-13 and alkaline  
phosphatase in syncytiotrophoblast**

**DOCTORAL DISSERTATION  
and  
PhD THESIS**

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**2006**

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## Introduction

### Galectin-13

Galectin-13 was isolated from human placenta and characterized in 1983. First it was identified as placental protein 13 (PP13). Later, cloning of PP13 were performed parallel by two research groups [1,2], and its sequence was deposited separately to the GenBank (AF117383, AY055826). By the expression of recombinant PP13, it became possible to perform more detailed functional studies on the protein. Conserved structural identity of PP13 to the members of the galectin family was found [1]. Several galectins have recently proved to be very closely related to galectin-13 [3,4]. Subsequently, computational 3D modelling based on its primary structure and homology to prototype galectins [5] revealed a characteristic “jellyroll” fold (deposited to Brookhaven Data Bank, Acc. No.: 1F87), a single conserved carbohydrate recognition domain (CRD) and predicted sugar binding capabilities of PP13, and therefore was designated as galectin-13 [6].

Galectins are a growing family of  $\beta$ -galactoside-binding lectins, which have been proved to have diverse biological functions, including regulation of cell adhesion, cell growth, and apoptosis [7-11]. Their evolutionarily highly conserved sequences have close similarity to each other among family members. These proteins are mainly localized in the cytoplasm, but they can be secreted, possibly through a nonclassical secretory pathway [12]. To date 14 mammalian galectins have been cloned and characterized [13]. Screening the databases of genomic DNA sequences and expressed sequence tags has revealed additional candidates for membership in the mammalian galectin family, as well as putative galectins in plants and viruses [9]. The structural and functional characteristics of galectins and their possible role in placental development and regulation pathways are receiving increased interest at present.

Galectin-13 was found to be comprised of two identical 16 kDa subunits held together by disulfide bonds, a phenomenon differing from the non-covalent dimerization of previously known prototype galectins and to have the lowest carbohydrate content (0.6%) of any known placental proteins [14]. It shared the highest homology to human eosinophil Charcot Leyden Crystal (CLC) protein / galectin-10 [15], and, like CLC, galectin-13 purified from human placenta showed weak lysophospholipase (LPL) activity [1], which was confirmed by  $^{31}\text{P}$  NMR. Since LPL activity of CLC protein / galectin-10 has recently

been assigned to its interaction with putative eosinophil LPLs or their known inhibitors [16], elucidation of intrinsic LPL activity, phospholipase (PL) or sugar binding activities of galectin-13 had to be reconsidered. Immunoaffinity purification and mass spectrometry (MS) studies indicated the binding of galectin-13 to proteins involved in phospholipid metabolism and cytoskeletal functions, but no intracellular LPL was detectably bound to it. On the other hand, intrinsic LPL activity for not only the purified protein, but also the bacterially expressed recombinant galectin-13 was confirmed. With sugar binding assays, the results of previous predictions on the sugar binding specificity of its CRD [6] were strongly underlined. Experiments revealed that N-acetyl-lactosamine, mannose and N-acetyl-glucosamine residues widely expressed in human placenta had the strongest binding affinity to both the purified and recombinant galectin-13, which also effectively agglutinated erythrocytes. Furthermore, reducing agents were shown to decrease its sugar-binding activity and abolish its haemagglutination. Phosphorylation sites were computed on galectin-13, and phosphorylation of the purified protein was empirically proved. Using affinity chromatography, PAGE, MALDI-TOF MS and PSD, annexin II and beta/gamma actin were identified as proteins specifically bound to galectin-13 in placenta and foetal hepatic cells. Perinuclear staining of the syncytiotrophoblasts showed its expression in these cells, while strong labelling of the syncytiotrophoblasts' brush border membrane confirmed its galectin-like externalization to the cell surface.

Although several data have been reported in connection with the structural characteristics of the protein [17], its possible function still remains a field of interest. According to data in literature, the involvement of the protein in cell death processes has been suggested, as it had been confirmed in cases of other galectin types.

Among the members of the family, galectin-1 and -3 have been extensively characterized, and the studies have suggested important roles for these proteins in cell-cell and cell-extracellular matrix interactions [18-21]. In addition, galectin-1 has been shown to induce apoptosis in activated T cells and thymocytes [22-24]. Induction of apoptosis has also been observed when galectin-1 was overexpressed in a prostate cancer cell line [25]. On the other hand, galectin-3 has been shown to be anti-apoptotic when ectopically expressed in Jurkat T lymphoma cells [26], in breast carcinoma cells [27-30] and human bladder carcinoma cells [31], although its proapoptotic role was also published [32]. Other investigated galectins, like galectin-7, galectin-8, galectin-9 and galectin-12 have also been proved to be involved in apoptotic events [13, 33-35].

Our group examined the possible role of galectin-13 in cell death. To investigate the function of galectin-13 in this process, we have generated and studied transient transfectants of differentiated U-937 cells to express this protein. Ask-1 phosphorylation occurred in cells overexpressing galectin-13. As it was also found in immunocytochemical studies on cell morphology, Taxol and hydrogen-peroxide treatment resulted in an advanced stage of apoptotic or necrotic state, compared to control transfected cells. Increased mitochondrial cytochrom-c release in galectin-13 overexpressing cells, as well as nuclear translocation of apoptosis-inducing factor (AIF) and endonuclease-G was detected. Further studies with Taxol and hydrogen-peroxide exposition were applied to confirm the sensibilising effect of galectin-13 on cell death. Main signal transduction pathways, such as Akt/PKB, Erk1/2, p38-MAPK, JNK/SAPK were examined by Western-blot techniques, so that we could determine, which members participated in the signaling induced by galectin-13. Effect of cyclosporin-A, caspase-3 inhibitor and inhibitors of the examined MAP kinase members strengthened the experiments, that p38-MAPK activation plays important role in Taxol-induced galectin-13 triggered apoptotic processes. At the same time, JNK/SAPK activation has an opposite, but mild effect in the presence of Taxol or hydrogen-peroxide. Cyclosporin-A exposition resulted in the highest increase in cell survival rate of galectin-13 overexpressing cells if treated with H<sub>2</sub>O<sub>2</sub>, but not in case of Taxol exposition. Finally, ROS generating systems were found not to be responsible for these events. Role of caspase-3 activation was also excluded concerning the results of immunocytochemical labeling with cell-permeable caspase-3 substrate and results of cell viability assays in the presence of caspase-3 inhibitor.

Summarizing our experiences, we found that galectin-13 expression resulted in increased cell death induced by further stimuli, suggesting that galectin-13 acts on a common point in the apoptotic signaling pathways and necrotic processes. Increased cell death caused by galectin-13 is independent of caspase-3 activation, but associated with mitochondrial cytochrome-c release. Galectin-13, in addition, heightens Ask-1 phosphorylation mediated p38-MAPK activation, especially after Taxol exposition.

With regard to our functional and immunomorphological results, moreover knowing its co-localization and specific binding to actin and annexin II, galectin-13 may have special haemostatic and immunobiological functions at the lining of the common foeto-maternal blood-spaces or developmental role in the placenta. Our new results suggest the possible participation of galectin-13 in apoptotic and necrotic processes of trophoblast cells and its contribution to the natural turnover of these cell types in the development, maturation and

aging of the placental tissues during pregnancy, as well as its predicted role in different pregnancy complications [36].

### **Alkaline phosphatase**

Alkaline phosphatase (AP) is known to be produced by the liver, bones, small intestine and kidneys, while different AP isoforms are also expressed by the placenta during pregnancy [37]. The placental isoforms are also called heat-stable AP (HSAP), as they are heat resistant at 60 °C, which property is a main criterion in distinguishing from other isoenzymes [38]. In early pregnancy, mostly tissue-unspecific AP isoenzyme is expressed in placentas reaching a peak value around 10 weeks of pregnancy. At the end of the second trimester, AP activity is mainly composed of term placental AP isoenzymes [37] (90 % of which is of P1 type, 10 % is of P2 type) produced by the syncytiotrophoblasts and appear in maternal serum between the 15-26<sup>th</sup> weeks of pregnancy [39]. Their plasma levels increase exponentially during gestation at a level three times higher than in non-pregnant women, and eliminate with a long half-life (7 days) postpartum [40]. Extreme increases in AP levels may be regarded as suspicion of bone, hepatic, endocrine, renal diseases, malignancies, drug treatment, but can be associated with heavy smoking or pregnancy as well (Table) [38,39,41].

**Table:** Differential diagnosis of elevated serum AP levels with regard to the origin of AP isoenzymes.

| <b>Tissue unspecific isoenzyme</b>   | <b>Placental isoenzyme</b>                                       | <b>Intestinal isoenzyme</b>              |
|--|--|--|
| <i>Normal tissue origin</i><br>Early placenta (<10 weeks)<br>Liver<br>Bone<br>Kidney | <i>Normal tissue origin</i><br>Term placenta<br>Uterus<br>Testis | <i>Normal tissue origin</i><br>Intestine |

|   |  |   |
|---|--|---|
| <p><i>Bone diseases</i></p> <ul style="list-style-type: none"> <li>Fracture</li> <li>Paget disease</li> <li>Rachitis</li> <li>Osteomalacia</li> <li>D-hypervitaminosis</li> <li>Osteogenesis imperfecta</li> <li>Osteoporosis</li> </ul> <p><i>Hepatic diseases</i></p> <ul style="list-style-type: none"> <li>Hepatitis</li> <li>Obstructive icterus</li> <li>Primary biliary cirrhosis</li> <li>Cholestasis</li> </ul> <p><i>Renal disease</i></p> <ul style="list-style-type: none"> <li>Renal failure</li> </ul> <p><i>Endocrine diseases</i></p> <ul style="list-style-type: none"> <li>Hyperthyreosis</li> <li>Hyperparathyreoidism</li> <li>Acromegaly</li> <li>Cushing syndrome</li> </ul> <p><i>Malignant tumours</i></p> <ul style="list-style-type: none"> <li>Hepatocellular carcinoma</li> <li>Bone sarcoma</li> <li>Lymphoma</li> <li>Bone and liver metastasis</li> </ul> <p><i>Drug treatment</i></p> <ul style="list-style-type: none"> <li>Antiepileptic therapy</li> </ul> | <p><i>Pregnancy related diseases</i></p> <ul style="list-style-type: none"> <li>IUGR</li> <li>Down syndrome</li> <li>Preeclampsia</li> </ul> <p><i>Malignant tumours</i></p> <ul style="list-style-type: none"> <li>Ovarian carcinoma</li> <li>Pancreas carcinoma</li> <li>Gastric carcinoma</li> </ul> <p><i>Hepatic disease</i></p> <ul style="list-style-type: none"> <li>Cholestasis</li> </ul> <p><i>Others</i></p> <ul style="list-style-type: none"> <li>Heavy smoking</li> </ul> | <p><i>Endocrine disease</i></p> <ul style="list-style-type: none"> <li>Diabetes mellitus</li> </ul> <p><i>Hepatic diseases</i></p> <ul style="list-style-type: none"> <li>Cirrhosis</li> <li>Gilbert-syndrome</li> </ul> <p><i>Renal diseases</i></p> <ul style="list-style-type: none"> <li>Renal failure</li> <li>Chronic renal disease</li> </ul> <p><i>Drug treatment</i></p> <ul style="list-style-type: none"> <li>Steroid therapy</li> </ul> |
|---|--|---|

Concerning, that the etiology of this experience is still unknown and no literature data could be found dealing with its cellular background, we decided to examine the biochemical and pathophysiologic events of this phenomenon.

Diagnosis of extremely high alkaline phosphatase (AP) level (3609 IU/l) was established in a 20-year old primigravida at the 37<sup>th</sup> gestational week. The phenomenon drew our attention to examine its histological and cellular origin. Immunohistochemistry and Western-blots using antibodies against AP, Ki-67, phospho-Akt, phospho-p44/42 MAPK / Erk1/2, phospho-GSK-3 $\beta$ , phospho-SAPK/JNK, total-Akt, total-GSK-3 and phospho-p38-MAPK were carried out on samples of index and control placentas at the same gestational stage. Compared to controls, staining of the index placenta resulted in minimal AP labelling of the brush border and remarkable positivity of the intervillous space. 8-10% of cytotrophoblastic proliferation was found in index placentas compared to the 1-2% in controls. Elevated levels of protein kinases known to play important role in cell

differentiation were also present in the index placenta. Based on these results, the proliferation and differentiation rate of the cytotrophoblasts were found to be 5 times higher in index samples than in controls. According to the most appropriate hypothesis, loss of the syncytial membranes in immature villi led to increased AP levels in maternal circulation and decreased AP staining of the placenta. Loss of the syncytium might also stimulate increased proliferation of villous cytotrophoblasts, which would then fuse and maintain the syncytium.

## **Materials and methods**

### **Galectin-13**

#### **Materials**

Galectin-13 antigen was prepared by Dr. Hans Bohn (Behringwerke AG, Marburg/Lahn, Germany). Anti-galectin-13 rabbit polyclonal antibody was developed by our team. We used anti-annexin II rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), fluorescein isothiocyanate (FITC) labelled anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and FITC labelled anti-rabbit IgG (BD Pharmingen, San Diego, CA, USA). We obtained WRL-68 human foetal hepatic cells (ATCC, Manassas, VA, USA); D<sub>2</sub>O (Isotec Inc., Miamisburg, OH, USA); pUC57-T vector (MBI Fermentas, St. Leon-Rot, Germany); pQE30 vector, M15 (pREP4) *E. coli* and Ni-NTA column (Qiagen Inc., Valencia, CA, USA); Protein A column (Affiland, Ans-Liege, Belgium); BCA reagent (Pierce Biotechnology Inc., Rockford, IL, USA); ECL chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK); DRAQ5 dye (Biostatus Ltd., Shepshed, UK); Universal Kit (Immunotech, Marseille, France); Pro-Q Diamond Phosphoprotein Gel Staining Kit (Molecular Probes, Eugene, OR, USA); trypsin (Promega GmbH, Mannheim, Germany); ZipTipC18 pipette tips (Millipore, Bedford, MA, USA). N-acetyl-D-lactosamine, L-fucose, galactose, glucose, lactose, maltose, mannose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine; cyanogen-bromide activated sepharose 4B, L-fucose-agarose, glucose-agarose, lactose-agarose, maltose-agarose, mannose-agarose, N-acetyl-D-galactosamine-agarose, N-acetyl-D-glucosamine-agarose; 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phospho-L-serin, L-phosphatidylinositol, L-phosphatidyl-ethanolamine; L- $\alpha$ -1-lysophosphatidylcholine, lysophosphatidylethanolamine, L- $\alpha$ -1-lysophospha-

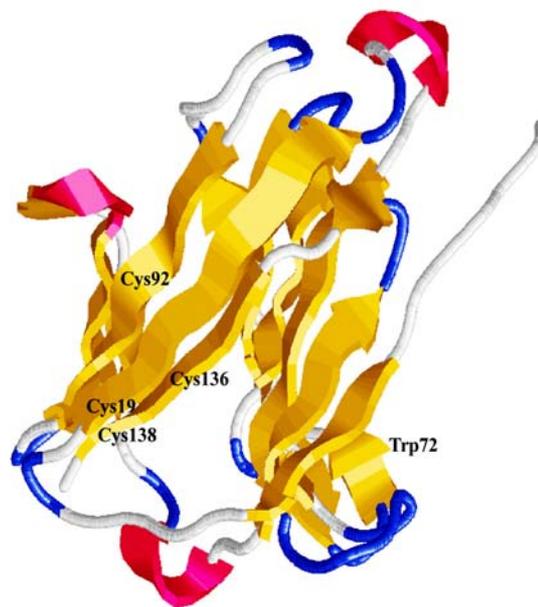
tidylinositol, L-  $\alpha$  -1-lysophosphatidyl-L-serin; isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG); antibiotic-antimycotic solution, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid) (HEPES), phenylmethylsulfonyl-fluoride (PMSF); horseradish peroxidase labeled anti-rabbit and anti-mouse IgGs were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Differentiated U-937 human macrophage cell line was cultured in DMEM medium supplemented with 10 % heat inactivated foetal calf serum (FCS) and 1% Antibiotic/Antimycotic solution (Life Technologies) under 5% CO<sub>2</sub> condition and 95% humidified air. Reagents for transfection experiments were also purchased from Sigma if not stated otherwise. Transient transfection was carried out by pcDNA3 vector (Invitrogen, Carlsbad, CA). For performing in vitro transfection of the cells, Lipofectamine-2000 (Invitrogen) was applied. Selection of transfected cells was carried out with geneticin-sulphate/G-418.

### Databank search

Galectin-13 cDNA and amino acid sequences were compared to various EST, genomic and protein databases by BLAST at NCBI (Bethesda, MD, USA) [42]. Multiple sequence alignments were carried out with CLUSTALW at EMB-net (Lausanne, CH) [43]. The PROSITE [44] and NetPhos [45] databases were searched for biologically significant patterns and putative phosphorylation sites.

The carbohydrate binding moiety and cystein residues probably involved in intermolecular cross-linking were localized on the 3D model of galectin-13 (PDB: 1F87) with RasMol [46].



### **Construction of bacterial galectin-13 expression plasmids**

Full length galectin-13 cDNA was isolated by the standard RACE method [47,48] using 4 µg of total placental RNA and specific primers. The resulting PCR fragments were inserted into pUC57-T cloning vector. Insert-containing clones were selected and sequenced by automated DNA sequencing at the Biological Services of the Weizmann Institute (Rehovot, Israel). Subsequently, the whole open reading frame of the cDNA containing the consensus Kozak sequence at its 5' end [49] was PCR amplified with (5'-CGATACGGATCCATGTCTTCTTTACCCGTGC-3') and (5'-TAAGTCGAGCTCATTGCAGACACACACTGAGG-3') primers. The resultant PCR product was cloned into the *Bam*HI and *Sac*I sites of the pQE30 expression vector.

### **Expression and purification of recombinant galectin-13**

The galectin-13/pQE30 expression vector was transformed into M15 (pREP4) *E. coli* host strain and the bacteria were induced with IPTG. The expressed protein was subsequently purified with Ni-NTA column in the presence of the 6-His-tag. The primary structure and purity of the recombinant protein was verified by sequence analysis [50] and by immunoblotting with both poly- and monoclonal anti-galectin-13 antibodies (Hybridoma Center of the Weizmann Institute).

### **Galectin-13 lysophospholipase and phospholipase activity detection by NMR**

Galectin-13 purified from placenta and bacterially expressed recombinant protein (20 µg each) was dissolved in 500 µl aqueous solutions (200 mM HEPES, 5.0 mM CaCl<sub>2</sub> and 130 mM NaCl, pH 7.4) of different 5.0 mg/ml lysophospholipids listed in materials. Aliquots without galectin-13 were used as controls. The solutions were prepared and stored at 37°C in 5 mm (outside diameter – o.d.) NMR-tubes and their <sup>31</sup>P NMR spectra were recorded at various time intervals. During NMR measurements a 2 mm O.D. insert tube filled with D<sub>2</sub>O was placed in the NMR tubes. To detect phospholipase activity of isolated and recombinant galectin-13, 7.2 mg/ml phospholipids listed in materials were used, and 25 µl Triton X-100 was added to the aliquots to enable dissolution of the substrate. <sup>31</sup>P NMR spectra were obtained on a Varian <sup>UNITY</sup>INOVA 400 WB spectrometer at 161.90 MHz at 37°C. 128 transients were acquired with proton decoupling using 30°C flip angle pulses with 3.4 s delays and a 0.6 s acquisition time, in order for the peak

integrals to represent the relative concentrations of the phosphorus-containing species. The chemical shifts were referred to the deuterium resonance frequency of the D<sub>2</sub>O in the insert tube. The relative concentrations (in molar fractions) of the species observed during the whole course of the study were determined by deconvolution of the spectra, using the routine built into the NMR software (Vnmr 6.1 B; Varian Inc., Palo Alto, CA, USA).

### **Galectin-13 sugar-binding assays**

Binding of recombinant galectin-13 to different sugars was studied essentially as described in other publication [51], but protein binding was followed by the endogenous fluorescence of galectin-13 (excitation at 280 nm, emission at 360 nm). 50 µg of the protein was dissolved in 200 µl sodium phosphate buffer (50 mM, pH 7.3, containing 0.15 M NaCl, 20 mM EDTA) and added to 50 µl activated sugar-coupled agarose beads as listed in materials. In parallel experiments, 1 mM DTT was also added to the mixture. The solutions were incubated in 0.5 ml microtubes at 37 °C for 1 hour with vigorous shaking. Tubes were then centrifuged at 7000 rpm for 20 s to sediment agarose beads. For quantification of unbound galectin-13, fluorescence of the supernatants was determined in a protein concentration range of 2-100 µg/ml, measured by an LS50B Perkin Elmer Luminescence Spectrometer (Shelton, CT, USA). For controls, uncoupled agarose beads (Sephacrose 2B) were used. After removing the unbound galectin-13, specifically bound proteins were eluted with different sugars in different concentrations (1 mM – 1 M) and fluorescence of the supernatants was measured by the same method. For positive control, galectin-13 (50 µg) was dissolved in buffer, for negative control only buffer was used.

### **Galectin-13 haemagglutination assay**

Lectin activity of both isolated and recombinant galectin-13 was determined by measurement of their capabilities to agglutinate human erythrocytes. Agglutination assays were performed in a 96-well microtiter plate with serial two-fold dilutions (0.21-200 µg/ml) of the proteins in PBS. Assays were also carried out by the addition of 1-1 mM DTT, mannose or N-acetyl-lactosamine to the mixtures. Samples (50µl) were gently mixed with 2% suspension of erythrocytes (50µl) and incubated at room temperature for 1 h. Agglutination activity was determined on the basis of the sedimentary state of the erythrocytes.

### **Galectin-13 dimerization assay**

For the detection of dimerization, recombinant galectin-13 was diluted (0.16-0.6 mg/ml) in Laemmli solution prepared with or without 10% (v/v) 2-mercaptoethanol and subjected to 12% (w/v) SDS-PAGE, then visualized by Coomassie staining. Protein bands were identified by subsequent MALDI-TOF mass spectrometry.

### **Pro-Q Diamond Phosphoprotein Gel staining**

20-20 µg isolated galectin-13, recombinant protein, ovalbumin (positive control) and BSA (negative control) were pretreated in reducing conditions, run on 15% SDS-PAGE and stained with Pro-Q Diamond Phosphoprotein Gel stain according to the manufacturer's protocol. For detecting phosphoproteins, the gel was visualized and photographed in UV light. For detecting its total protein content, Coomassie staining was applied.

### **Cell culture**

WRL-68 cells were grown on 100-mm dishes in standard DMEM containing 1% antibiotic-antimycotic solution, supplemented with 10% FCS under 5% CO<sub>2</sub> condition and 95% humidified air at 37°C. Cells were harvested and low-speed centrifuged (2000 g), then the pellet was dispersed by vortexing in lysis buffer (50 mM Tris pH 7.4, 1 mM PMSF) for 10 min at 4 °C. After further cell disruption in a Teflon/glass homogenizer, the homogenate was pelleted, and the supernatant was coupled to cyanogen-bromide activated Sepharose 4B by the instructions of the manufacturer.

### **Tissue preparations**

100 mg tissue blocks from a term human placenta obtained from Histopathology Ltd. (Pecs, Hungary) were homogenized in lysis buffer (50 mM Tris pH 7.4, 1 mM PMSF) for 10 min at 4 °C in a Teflon/glass homogenizer. After pelleting the homogenates, supernatants were either coupled to cyanogen-bromide activated, galectin-13-bound Sepharose 4B for immunoaffinity purification, or measured by BCA reagent and equalized for 1 mg/ml protein content in 2x Laemmli solution for Western blotting. Other parts of the placenta were formalin-fixed, paraffin-embedded, cut for 4 µm sections, mounted on slides, dried at 37 °C overnight, dewaxed and rehydrated for immunohistochemistry and immunofluorescence confocal microscopy.

### **Affinity purification of galectin-13 bound proteins**

Both isolated and recombinant galectin-13 were coupled to cyanogen-bromide activated Sepharose 4B and incubated with protein extracts from human placenta or WRL-68 foetal hepatic cells at 24 °C for 1 h. For controls, samples were incubated with uncoupled Sepharose 4B. Gels were washed three times with 20 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl) followed by four rinses with 20 mM Tris-HCl buffer (pH 7.4) to remove unbound proteins. Specifically bound proteins were removed by an equal volume of 2x Laemmli buffer, separated by 15 % SDS-PAGE and visualized by Coomassie stain.

### **Protein identification by mass spectrometry**

Bands of interest in Coomassie stained galectin-13 (isolated and recombinant), as well as galectin-13 bound and eluted protein extracts were excised from the gels, reduced, alkylated and in-gel digested with trypsin as described in literature [52]. Proteins were identified by a combination of MALDI-TOF MS peptide mapping and MALDI-PSD MS sequencing. The digests were purified with ZipTipC18 pipette tips with a saturated aqueous solution of 2,5-dihydroxybenzoic acid matrix (ratio of 1:1). A Bruker Reflex IV MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) was employed for peptide mass mapping in positive ion reflector mode with delayed extraction. The monoisotopic masses for all peptide ion signals in the acquired spectra were determined and used for database searching against a non-redundant database (NCBI, Bethesda, MD, USA) using MS Fit program (UCSF, San Francisco, CA, USA) [53]. Primary structure of tryptic peptide ions was confirmed by PSD MS sequencing.

### **SDS-PAGE / Western blot**

10-10 ng isolated and recombinant galectin-13, and 10 µg of human placental protein extract was subjected to 15% (w/v) SDS-PAGE followed by immunoblotting with poly- or monoclonal anti-galectin-13 antibodies and horseradish peroxidase labeled secondary IgGs as described in a previous article [54]. Protein bands were revealed by ECL chemiluminescence system.

### **Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue sections were incubated either with mono- or polyclonal galectin-13 antibodies. Immunostaining was carried out according to the streptavidin-biotin-peroxidase technique using Universal Kit [55]. Control sections were

incubated only with secondary IgGs. Visual evaluation of hematoxylin-counterstained slides was performed with Olympus BX50 light microscope with incorporated photography system (Hamburg, Germany).

### **Immunofluorescence confocal microscopy**

Paraffin embedded tissue sections were deparaffinated and treated with either mono- or polyclonal galectin-13 antibodies followed by FITC labelled secondary anti-mouse or anti-rabbit IgGs and 20  $\mu$ M DRAQ5 dye nucleus labelling in PBS containing 0.1-0.1% (v/v) saponin and BSA. To visualize the localization of annexin II, anti-annexin II primary and FITC labelled secondary IgGs were used. Control sections were incubated with only secondary IgGs, antigen depletion was carried out on distinct slides. Fluorescence was scanned with a Bio-Rad MRC-1024ES laser confocal attachment (Herefordshire, UK) mounted on Nikon Eclipse TE-300 inverted microscope (Kingstone, UK).

### **Experiments with transfected cells**

#### **Cloning and transfection**

The galectin-13 cDNA was originally cloned from transformed host strain cells, described in our previous article (galectin-13/pQE30 expression vector containing M15 /pREP4/ E. coli), using primers with BamHI and XhoI restriction sites. Either the galectin-13 sequence, or the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) were digested with BamHI and XhoI restriction endonucleases. After the insert had been ligated into pcDNA3, the success of ligation was confirmed by a probe of PCR and agarose gel electrophoresis. The resulting construct was applied for transfection methods. Differentiated U-937 cells were transfected with the recombinant plasmid pcDNA3-galectin-13 or pcDNA3 alone (as control), applying Lipofectamine-2000 according to the manufacturer's guideline. Briefly, cells were grown to approximately 70% confluence in plastic Petri-dishes, washed twice with PBS, and overlaid with 5 ml of DMEM without FCS, containing the plasmid (1  $\mu$ g/ml) and Lipofectamine-2000. Five hours later, the media was completed with 5 ml of DMEM – FCS. Cells were selected by G-418/geneticin (500  $\mu$ g/ml), after the sensitivity of original U-937 cells for the selecting agent had been determined. Transient transfection of the cells could be maintained by this method. We studied the consequences of several stress effects on galectin-13 overexpressing and control cells by MTT - assay in 96-well plates.

### **Cell Viability Assay**

Transfected cells (pcDNA-galectin-13-gene construct or pcDNA alone) were seeded into 96-well plates at a starting density of  $10^4$  cell/well and cultured overnight, before the following treatments have been applied: 0.15 mM H<sub>2</sub>O<sub>2</sub>, 0.3 mM H<sub>2</sub>O<sub>2</sub>, 0.45 mM H<sub>2</sub>O<sub>2</sub>, 10 nM Taxol, 100 nM Taxol, 1 μM Taxol, 1 μM lipopolysaccharide, 10 μM lipopolysaccharide, 1 μM etoposide, 10 μM etoposide. After the culture period, the media were removed and replaced with DMEM containing an appropriate amount of the MTT solution (Chemicon Inc., El Segundo, CA) to each well for 4 hours. The MTT reaction was terminated by adding HCl to the medium at a final concentration of 10 mM. The amount of water-insoluble blue formazan dye formed from MTT was proportional to the number of living cells and was determined with an Anthos Labtech 200 enzyme-linked immunosorbent assay reader at 550 nm wavelength after dissolving the blue formazan precipitate in 10% SDS. All experiments were run in at least four parallels and repeated three times.

### **Examination of the effect of H<sub>2</sub>O<sub>2</sub>- and Taxol-exposition on galectin-13 overexpressing cells. Analysis of MAPK-phosphorylation in Western blot, determination of MAPK-activation profile (24 hour experiment)**

Based on the results of MTT-assay, following expositions were carried out with H<sub>2</sub>O<sub>2</sub> and Taxol, as they had had the most significant differences in their effect on index and control cells.

A 24-hour experiment was performed, in which either the galectin-13 overexpressing, or the control cells were divided into 9 groups. First of them was the reference (0 hour of exposition), while from the second to the ninth group were treated with 0.3 mM hydrogen-peroxide during 3, 6, 9, 12, 15, 18, 21 and 24 hours. The same procedure was carried out with 100 nM Taxol exposition.

The cells were grown in 6 well-plates and stimulated at  $5 \times 10^5$  cells/ml in DMEM-FCS by adding the indicated amounts of H<sub>2</sub>O<sub>2</sub> or Taxol. After the required time of incubation the culture media was eliminated and activation was stopped by addition of the same volume of ice cold lysis buffer, supplemented with the phosphatase inhibitor Na<sub>3</sub>VO<sub>4</sub> (0.5 mM). The cells were kept on ice and lysed by ultrasound homogenisation. Protein fraction was isolated by centrifugation at 10 000 x g for 15 min. Supernatants were collected, and their protein contents were measured using the BioRad assay and equalised in Laemmli sample buffer. Protein extracts (10 μg each) were loaded and separated by 12% sodium

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane. Membranes were blocked using TBS (tris buffered saline) solution containing 0.05% Tween-20 and 4 % milk, washed three times with TBS/Tween-20 (0.05%) and subsequently probed with rabbit polyclonal IgGs against the following antigens: phospho-Akt/PKB (phospho-protein kinase B, Ser473), phospho-Erk-1/2 (p44/42 mitogen activated protein kinase/extracellular signal regulated kinase-1/2, Thr202/Tyr204), phospho-SAPK/JNK (phospho-stress activated protein kinase/c-Jun N-terminal kinase, Thr183/Thr185), phospho-p38-MAPK (Thr180/Tyr182), and total-Akt (Cell Signaling Technology Inc, Beverly, Massachusetts, USA). Horseradish peroxidase labelled anti-rabbit IgG (Sigma-Aldrich Co, St Louis, Missouri, USA) was used as secondary antibody. Protein bands were revealed by the ECL chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

### **Studies on the effect of MAPK inhibitors', caspase-3 inhibitor and cyclosporin-A on cell viability**

Galectin-13-pcDNA3 and empty pcDNA3 vector transfected cells were seeded into 96-well plates at a starting density of  $10^4$  cells/well and cultured overnight before  $H_2O_2$ , Taxol were added to the medium at a concentration and composition indicated in the figure legends. After the culture period, the media were removed and replaced with DMEM containing the following inhibitor agents: LY294002 (PI3K-Akt pathway inhibitor), PD98059 (Erk pathway inhibitor), SB203580 (p38-MAPK inhibitor), SP600125 (inhibitor of JNK/SAPK) and caspase-3 inhibitor /Calbiochem/. All of these inhibitors were applied in 10  $\mu$ M concentration. Cyclosporin-A was used in a final concentration of 2  $\mu$ M. After 1 hour pretreatment with the previously described inhibitors, 0.3 mM  $H_2O_2$  was added to the cell cultures for 12 hours or 10 nM Taxol for 24 hours. Following to the required time of exposition, cell viability assays (MTT) were performed on each plates.

### **Immunocytochemical studies on cell morphology**

Galectin-13 overexpressing and control cells have been grown in a 6-well plate containing coverslips. 3 -3 samples were created from both transfectants. After reaching a confluence at about 70 %, 1-1 of each group remained untreated, 1-1 of them was treated with 0.3 mM  $H_2O_2$  for 12 hours and 1-1 of each cell samples were subjected to 10 nM Taxol for 24 hours. After subjecting the cells to the exposition, coverslips were rinsed twice in PBS.

Finally, cells were fixed by adding 4% formaline solution and were stained by the Actin-cytoskeleton Focal Adhesion Staining Kit (Chemicon<sup>®</sup> International), so as to visualize and compare the morphology and architecture of galectin-13 overexpressing and control cells. For vinculin staining, Alexa Fluor 546-conjugated goat anti-mouse IgG was purchased (Molecular Probes, Invitrogen) to complete the kit. Samples were embedded and examined with an Olympus BX61 fluorescent microscope equipped with a ColorView CCD camera and analysis software was used with 60x objective.

### **Examination of cell death**

#### **Detection of mitochondrial protein release and changes in cell signaling pathways**

Presence of AIF (apoptosis inducing factor) in mitochondrium, cytosol and nucleus, and cytochrom-c release were examined with Western-blot in galectin-13 overexpressing cells and controls. Both cell types were divided into three groups: one without any treatment, the second is exposed to H<sub>2</sub>O<sub>2</sub> (0.3 mM, 12 hours) and the third is exposed to Taxol (10 nM, 24 hours). 2 x 10<sup>6</sup> U-937 cells transfected by galectin-13/pcDNA3 or pcDNA3 empty vectors were treated in plastic Petri-dishes. After the exposition the cells were collected in a chilled lysis buffer of 0.5 mM sodium metavanadate, 1mM EDTA, and protease inhibitor mixture in phosphate-buffered saline. Nuclear, mitochondrial and cytosolic cell fractions were isolated. The proteins of each fraction were precipitated by trichloroacetic acid, washed three times with -20 °C acetone, and subjected to SDS-PAGE. Equal amounts of proteins were separated by a 12% polyacrilamid gel for the detection of AIF and by an 18% polyacrilamid gel for the detection of cytochrome-c. Western-blot was carried out as described previously.

#### **Measurement of reactive oxygen species (ROS)- production**

Based on the hypothesis that galectin-13 may play a role in apoptotic cell death, production of reactive oxygen species were measured in galectin-13 overexpressing and control U-937 cells, using C400-assay / 5-(and 6)-carboxy-2'-7'-dichloro-dihydro-fluorescein-diacetate/. Cells were seeded into 96-well plates at a starting density of 10<sup>4</sup> cell/well and cultured overnight. Either galectin-13 transfectants, or control cells were divided into five groups. One of each were without any treatment, four of them were subjected to 0.15 mM H<sub>2</sub>O<sub>2</sub>, 0,45 mM H<sub>2</sub>O<sub>2</sub>, 10 nM Taxol and 100 nM Taxol for 3 hours. At the same time, we supplied the experiment with the appropriate inhibitors of the

following ROS-generating enzymes: cyclooxygenase (indomethacin), cytochrome P-450 (metyrapone), nitric oxide synthase (NG-methyl-L-arginine), NADPH-oxidase (iodonium diphenyl), xanthine-oxidase (allopurinol), ribonucleotide-reductase (hydroxyurea) /Sigma/. Measurements were carried out in a 96-well plate by Anthos Labtech 200 enzyme-linked immunosorbent assay reader at 485 nm excitation and 555 nm emission wavelengths.

### **Immunocytochemical detection of Ask-1 phosphorylation/activation in apoptosis**

Galectin-13 overexpressing and control transfected cells were grown in DMEM-FCS medium onto a covering glass plate until reaching an approximately 70% confluence in a 6-well plate. 3 -3 samples were created from both transfectants. 1-1 of each group remained untreated, 1-1 of them was treated with 0.3 mM H<sub>2</sub>O<sub>2</sub> and 1-1 of each cell samples were subjected to 10 nM Taxol for 12 hours. After the exposition, immunocytochemical staining of phospho-Ask-1 proapoptotic protein in pcDNA3-galectin-13 construct containing U-937 cells and simple pcDNA3-transfected control U-937 cells were carried out by the following protocol: cells were washed three times with 1X PBS (phosphate buffered saline), permeabilized with 1X PBS supplemented with 0.1% Triton X-100, blocked with 2 % PBS-BSA solution, followed by a labelling with phospho-Ask-1 (Ser83) antibody (Cell Signaling Technologies), stained by FITC polyclonal anti-rabbit IgG (Becton Dickinson Co.), as secondary antibody. Nuclei were labelled with Hoechst-stain. After washing with 1X PBS, cells were visualized by Olympus BX61 fluorescent microscope equipped with a ColorView CCD camera and analysis software.

### **Detection of cell death by propidium-iodide/FITC-annexin-V staining**

Simple pcDNA3-transfected control U-937 cells and pcDNA3-galectin-13 construct containing U-937 cells were seeded on coverslips as described in previous experiments and the classical propidium-iodide/annexin-V staining was performed in order to prove the cell death enhancing effect of galectin-13. Samples were created according to the description in figure legends. Cells were visualized by the above mentioned fluorescent microscope and analysis software.

### **Measurement of caspase-3 and -7 activity**

The aim was to determine the possible role of caspase-3 and -7 in galectin-13 mediated cell death. Galectin-13 overexpressing and control transfectants were seeded at  $1 \times 10^5$  cells/well in a 6-well plate containing coverslips. Groups of samples were created and treated as it is described in previous experiments (see legend to the figure). All samples were stained with the Magic Red™ Caspase Detection Kit /caspase 3 and 7 MR-(DEVD)<sub>2</sub> / (Immunochemistry Technologies, LLC) in order to determine caspase-3 and -7 activity.

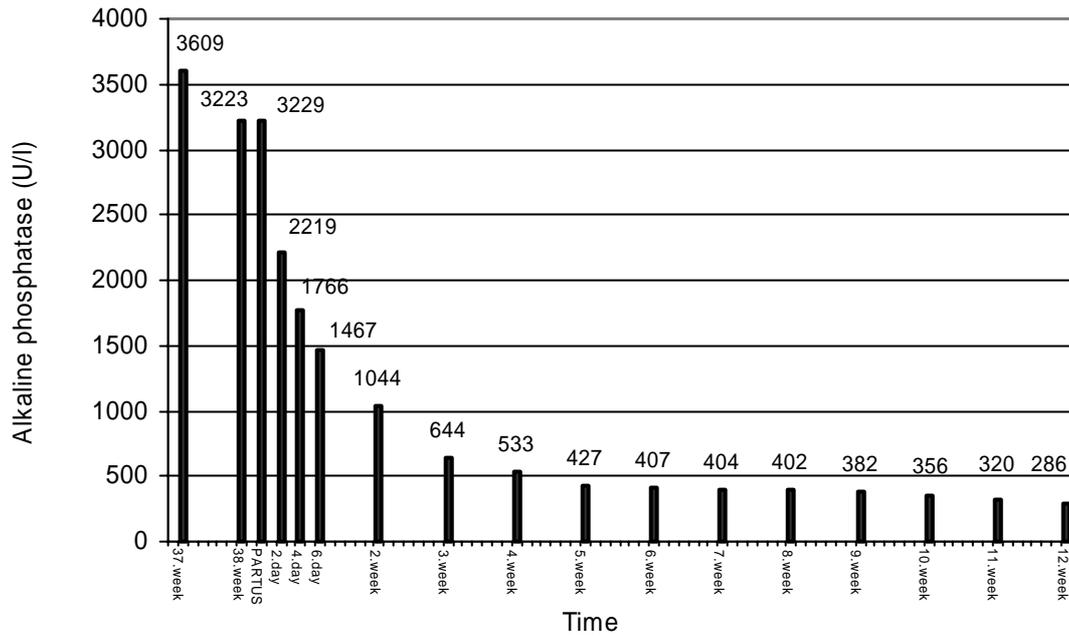
### **Statistical evaluation**

Values in the figures and text were expressed as mean  $\pm$  SEM of n observations. Statistical analysis was performed by analysis of variance followed by student T-test and chi-square test.  $P < 0.05$  was considered to be statistically significant.

## **Alkaline phosphatase**

### **Clinical data**

A 20-year old primigravida was admitted to the clinic because of oedema and suspicion of preeclampsia on the 37<sup>th</sup> gestational week. After general examination and precise observation, extremely elevated serum alkaline phosphatase (AP) level was detected (3609 U/l). Blood pressure was normal, proteinuria did not occur and the patient had no complaint. Laboratory tests presented normal results of blood parameters, renal, hepatic and endocrine functions compared to normal references. No systematic immune disease could be explored. Markers of ovarian tumours (CA-125, CEA, CA-19-9) did not show pathological level. Bone associated AP fraction was determined merely at 97 U/l. Electrophoresis assay of total AP by the Hydragel Protein kit (Sebia, Issy-les-Moulineaux, France) showed the presence of 65.9 % (2128 U/l) P1 and 30.1 % (972 U/l) P2 placental isozyme. On the 38<sup>th</sup> week of pregnancy a mature newborn girl was born by vaginal labour with a weight of 3,200g and an Apgar-score of 9/10. The 730g placenta was without any macroscopic abnormality or infarction. hCG level declined quickly after birth, while the level of AP decreased exponentially during the following weeks and reached normal level 12 weeks postpartum (Figure-1).



**Figure 1.** Changes in total serum AP levels during pregnancy and postpartum.

### Histopathology and immunohistochemistry

Samples were obtained from the index case and from normal term placentas (n=5). Tissue blocks were routinely fixed in formalin and embedded in paraffin. Full thickness representative blocks were taken from three different central-paracentral areas of each placenta and an en-face block of the maternal surface was cut. Further blocks from the umbilical cord and membranes were sampled for routine examination only. Four  $\mu\text{m}$  consecutive serial sections of the same blocks were cut and prepared also for immunostaining. Tissue samples were stained with haematoxylin-eosin for histopathological examinations, or with monoclonal IgG antibodies against cell proliferation marker Ki-67 (Histopathology, Pecs, Hungary) and placental AP (Lab Vision, Fremont, California) for immunohistochemical evaluations. Immunostaining was performed by the streptavidin-biotin-immunoperoxidase technique, with  $\text{H}_2\text{O}_2$ /3-amino-9-ethylcarbazole (AEC) development using Universal Kit (Immunotech, Marseille, France).

## **SDS-PAGE and chemiluminescence Western blot analysis**

Explants (100 mg each) were taken from five different areas of normal and index placental tissues, and were homogenized on ice in 10 ml of lysate buffer (pH 7.5, 50mM Tris, 1mM PMSF). Homogenates were centrifuged at 4,000g for 5 minutes, supernatants were collected, their protein contents were measured using the BioRad assay and equalized in Laemmli sample buffer. Protein extracts (10 µg each) were loaded and separated by 12% SDS-PAGE. Western blot analyses were performed applying phospho-Akt, phospho-p44/42 MAPK / Erk1/2, phospho-GSK 3β, phospho-SAPK/JNK, total-Akt and total-GSK-3 rabbit polyclonal IgGs, and mouse monoclonal phospho-p38-MAPK IgG (Cell Signaling Technology, Inc., Beverly, MA, USA). As secondary antibodies, horseradish peroxidase labelled goat anti-rabbit and anti-mouse IgGs (Sigma-Aldrich Co., St. Louis, MO, USA) were used. Protein bands were revealed by ECL chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). It was followed by quantitative densitometric analysis of the bands with Scion Image for Windows and ImageJ softwares.

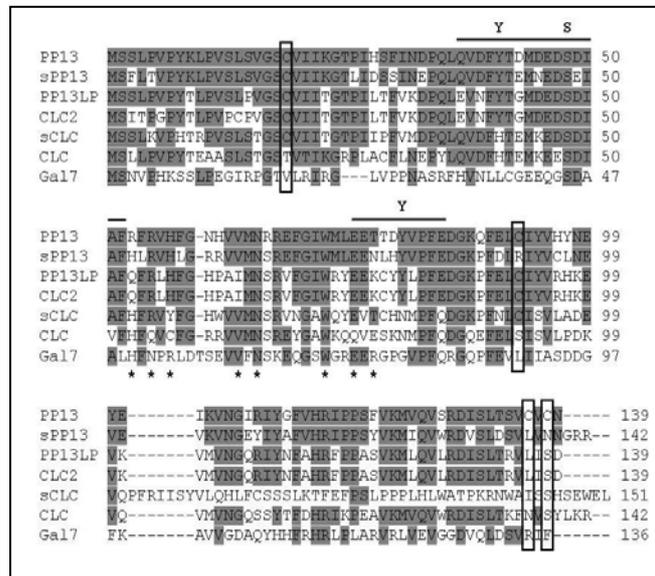
## **Results**

### **Galectin-13**

Galectin-13 is a member of a new subfamily among prototype galectins. By GenBank search of related EST sequences, it could be assumed that galectin-13 and its EST sequence appears in numerous fetal and adult tissues (fetal liver and spleen, adult brain, eye, mammary gland, skin, testis, cervix - NCBI database; Unigene), and gene expression array-based results also suggested the presence of galectin-13 transcripts in several normal cells or malignancies (macrophage, breast carcinoma, essential thrombocythemia, acute myeloblastic leukemia cells, natural killer cell, Kaposi sarcoma-associated herpesvirus infection of primary human dermal endothelial cells, pituitary adenoma subtypes, prostate adenocarcinoma, malignant melanoma cell lines, colon cancer, etc. - NCBI database; Geo Profils) as well. Galectin-13 gene mapped to chromosome 19 (19q13.1) in the close vicinity of genes of four known (galectin-10 [56], galectin-7 [57], galectin-4 [58] and placental protein 13-like protein [3]) and three putative (“similar to placental protein 13” -

Locus: XP\_086001 / AC005515-I [4], “unnamed protein” - Locus: BAC85631 / AC005515-II [4] and “Charcot-Leyden Crystal 2 protein” - Locus: AAP97241) galectins at 19q13.1-13.2 with similar exon structures, indicating their common genetic origin.

Galectin-13 was found to have close relation to the predominantly placental expressed “similar to placental protein 13” (69% identity, 80% similarity) and placental protein 13-like protein (68% identity, 79% similarity) as well as CLC protein (56% identity, 69% similarity). The putative “Charcot-Leyden Crystal 2 protein” and “unnamed protein” also had considerably high relation to galectin-13.



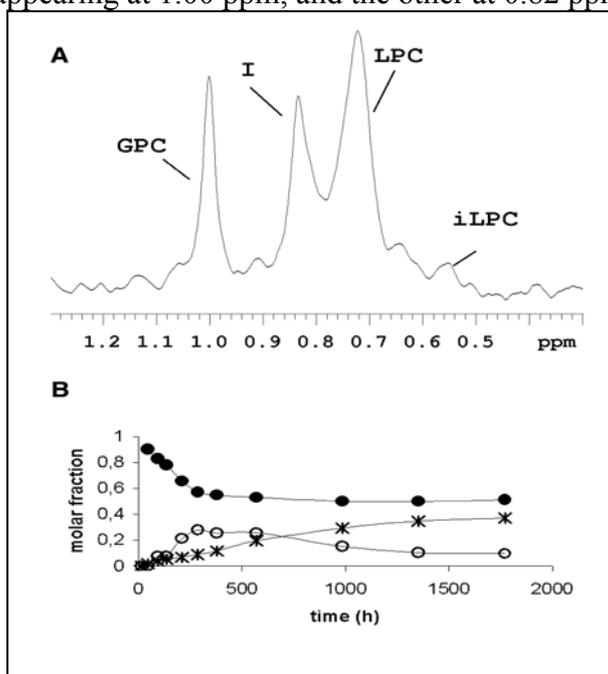
Putative serine and tyrosine kinase phosphorylation sites localized on the outer surface of galectin-13 were predicted at positions 44-52 (Ser48), 37-45 (Tyr41) and 76-84 (Tyr80) by computations. With RasMol, four cystein residues were revealed on the surface of galectin-13. By CLUSTALW alignments, Cys136 and Cys138 on beta-sheet F1 were found to be missing from all homologues. Cys19 and Cys92 on beta-sheets F2 and F3 were missing from distant homologues, but some of the newly described closest homologues contained them.

### Galectin-13 possesses weak endogenous LPLA activity

For both isolated and recombinant galectin-13, the highest degree of transformation was found for L- $\alpha$ -lysophosphatidylcholine (1-acyl-glycero-3-phosphorylcholine, LPC); other lysophospholipids showed at most 5% (molar) transformation during the same period (data not shown). In the course of LPC transformation four species could be distinguished and quantified in the  $^{31}\text{P}$  NMR spectra (A), and their relative concentrations showed similar time-dependence (see data for recombinant protein in(B); however, the reaction rates varied in the three solutions (isolated galectin-13, recombinant protein and control). The peak at 0.72 ppm could be assigned to the starting material, which was involved in an isomerization equilibrium with 2-acyl-glycero-3-phosphorylcholine (iLPC) ( $\delta=0.56$  ppm)

[59], independent from the presence of galectin-13 proteins. In a slower reaction, LPC was transformed into two other species, one appearing at 1.00 ppm, and the other at 0.82 ppm.

The former signal could be assigned to glycerol-3-phosphorylcholine (GPC) based on its chemical shift [1, 59-61]. The relative concentrations of the three major species ( $\delta = 1.00$ , 0.82 and 0.72 respectively), expressed in molar fractions, are shown in (B). The relative concentration of iLPC fluctuated between 0.10 and the limit of quantitation over the whole course of the reactions, roughly following the change of LPC (data not shown).

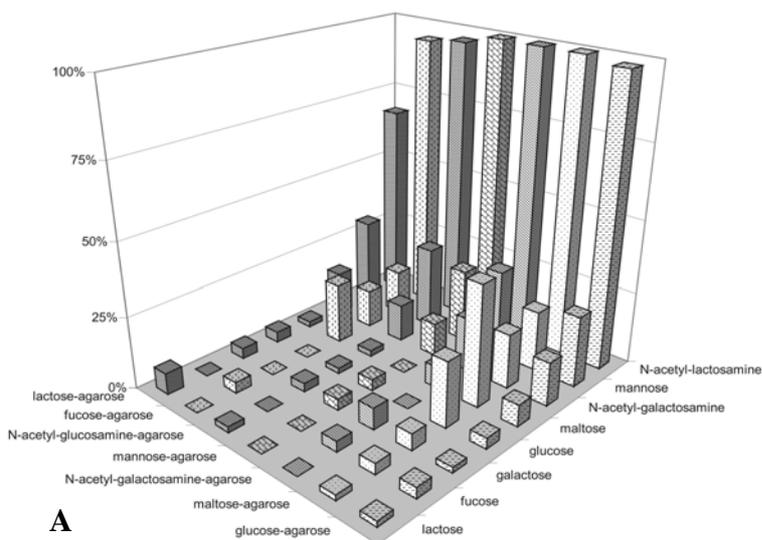


The kinetics of the transformation of LPC (●) could not be exactly described by classical models. However, the reaction appeared to move toward an equilibrium, judged by the time-dependence of the relative concentrations of the major species. The species appearing at 0.82 ppm might well be an intermediate in the transformation (○), since its molar fraction increased in the first period and decreased after reaching a maximum value. Attempts to identify this presumed intermediate are underway. Determination of the enzymatic activities was difficult, since the concentrations of both the intermediates and the products remained under the limit of quantitation for several tens of hours. However, a rough estimate could be made by the first spectra showing GPC (x) in quantifiable concentrations: isolated galectin-13 showed 4.8 mol% transformation in 306 h, recombinant galectin-13 gave 4.5 mol% in 210 h whereas control samples showed 1.1 mol% in 272 h. In terms of specific activity, these data read as 0.69, 0.94 and 0.18 nmol/min/mg, respectively, whereas approximately 1300  $\mu$ mol/min/mg was found for human brain lysophospholipase (LPL) [62] and 2.5  $\mu$ mol/min/mg for a lysophospholipase isolated from human amniotic membrane [63]. Phospholipase (PL) activity of isolated and recombinant galectin-13 was tested analogously, using phospholipids as substrates. No change could be observed in <sup>31</sup>P NMR spectra for any phospholipids, thus neither the isolated nor the recombinant protein appeared to possess detectable PL activity.

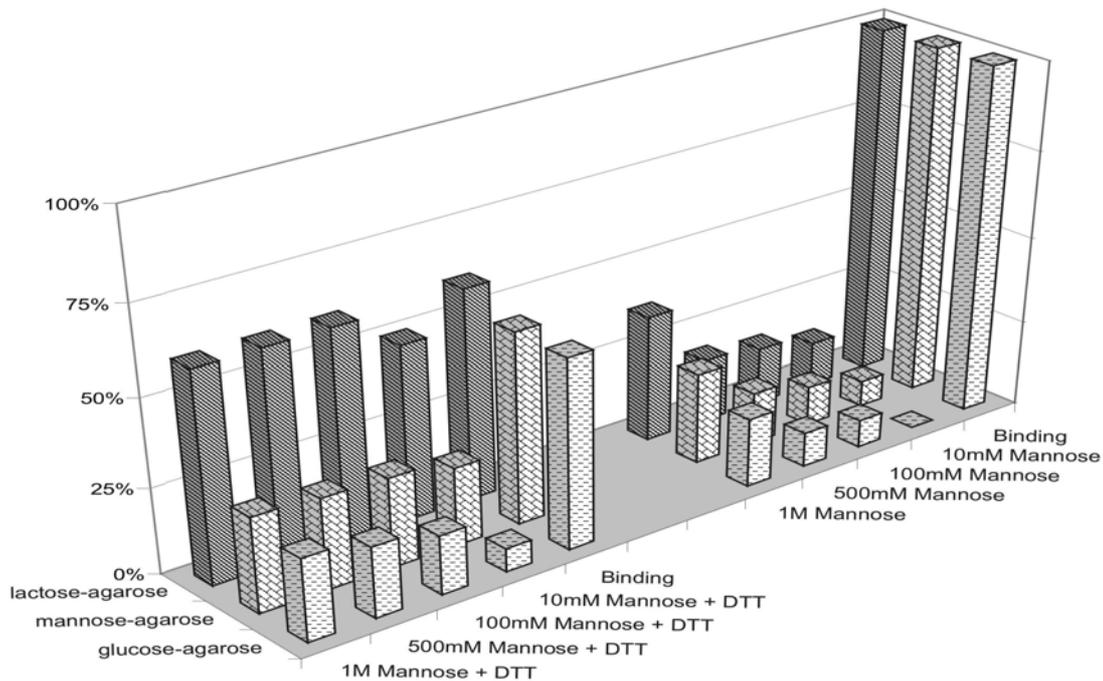
### Galectin-13 has strong sugar binding capabilities

Non-modified agarose beads (Sephadex 2B) did not bind galectin-13 at all, while all types of sugar-coupled agarose beads bound more than 95% of galectin-13 after 1 h incubation. Different sugars (1mM – 1 M) eluted the protein from various sugar-coupled agarose in different manners, with the following elution capacity: N-acetyl-lactosamine > mannose > N-acetyl-galactosamine > maltose > glucose > galactose > fucose > lactose (**A**).

In 1 M concentration, N-acetyl-lactosamine had significantly the highest efficacy (95-100%) to elute galectin-13 from all kinds of beads, while mannose was less effective, having an elution capacity between 15-30%. On average, N-acetyl-galactosamine was the third most effective to specifically compete with galectin-13 binding (12-19%).



The elution capacities for other sugars were determined to be below 8% in the following order: maltose (0-8%), glucose (0-4%), galactose (0-7%), fucose (0-4%), lactose (0-2%). These latter sugars had higher elution efficacy only in some special combinations: maltose / fucose-agarose (21%) and maltose-agarose (42%); glucose / maltose-agarose (23%); lactose / lactose-agarose (7%) (**A**). In the presence of 1 mM DTT during the 1 h binding period, approximately half of galectin-13 was bound to different sugar-coupled agaroses (e.g.: lactose-agarose: 60 %, glucose- and mannose-agarose: 55-55 %), and the elution of specifically bound galectin-13 with different sugars was four-times more effective compared to non-reducing conditions. 100 mM mannose eluted 31-100 % of galectin-13 from glucose-, mannose- or lactose-agarose, while without the presence of DTT the elution was only 8-16 % (**B**). The order of the elution capacity of the different sugars for galectin-13 from the various sugar-coupled agaroses was the same in reducing and non-reducing conditions, but in presence of DTT, sugar elution of specifically bound galectin-13 from sugar-coupled agaroses was significantly higher. 100 mM mannose eluted all bound galectin-13 from lactose-agarose, 50 % from mannose-agarose and 43 % from glucose-agarose.



**B**

### Galectin-13 possesses lectin activity

Lectin activity of isolated and recombinant galectin-13 was confirmed by measurements of their agglutination capabilities of human erythrocytes. In non-reducing conditions, very small amounts of galectin-13 induced haemagglutination, and strong agglutination was detected at and above 50  $\mu\text{g/ml}$  applied protein

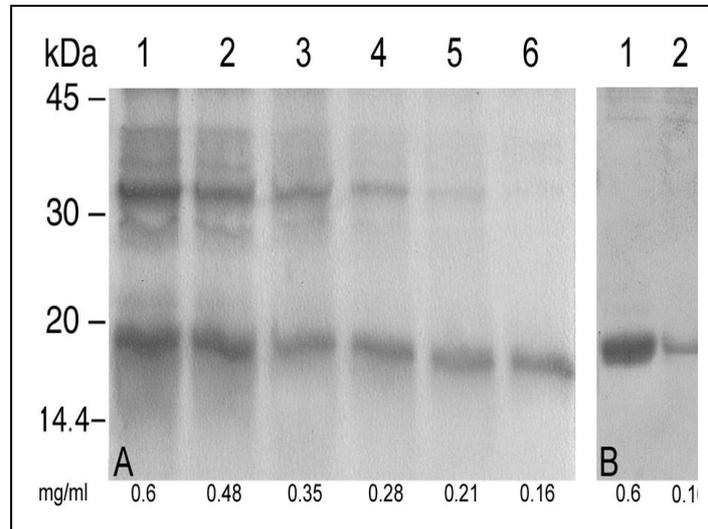
| 200 | 100 | 50 | 25 | 12.5 | 6.3 | 3.3 | 1.7 | 0.8 | 0.4 | $\mu\text{g/ml}$                  |
|-----|-----|----|----|------|-----|-----|-----|-----|-----|-----------------------------------|
|     |     |    |    |      |     |     |     |     |     | PP13-B                            |
|     |     |    |    |      |     |     |     |     |     | PP13-R                            |
|     |     |    |    |      |     |     |     |     |     | PP13-R + 1mM DTT                  |
|     |     |    |    |      |     |     |     |     |     | PP13-R + 1mM mannose              |
|     |     |    |    |      |     |     |     |     |     | PP13-R + 1mM N-acetyl-lactosamine |
|     |     |    |    |      |     |     |     |     |     | Control                           |

concentrations, which was very similar to the phenomenon seen in cases of other galectins [64]. The pattern and effectiveness of both isolated and recombinant protein were identical in agglutination of erythrocytes. However, no haemagglutination occurred in reducing conditions with the addition of 1 mM DTT to the mixture. Different sugars had also an inhibitory effect on haemagglutination capabilities of galectin-13. Approximately at and above concentrations of 1-1 mM N-acetyl-lactosamine and mannose, previously found to be the best ligands of the protein, abolished its haemagglutination activity.

### Galectin-13 dimerizes via disulphide bonds

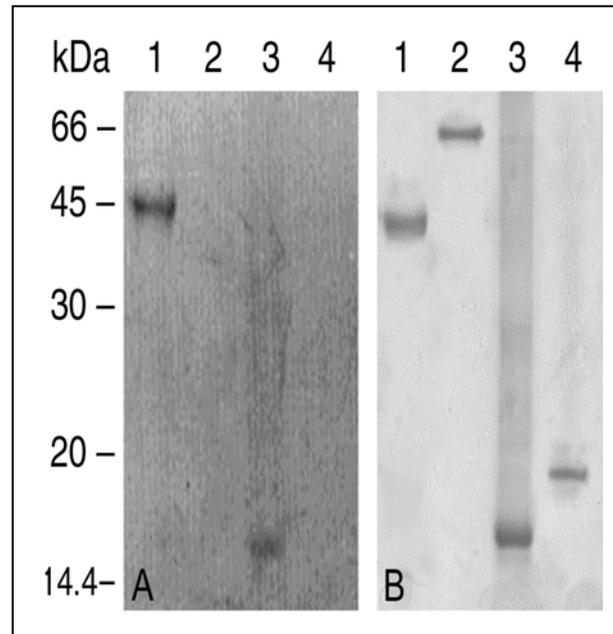
Galectins were known to be dimerized by non-covalent interactions [4,5]. Due to earlier data [14], as well as in our experiments, galectin-13 turned out to be composed of two identical subunits held together by disulphide bonds.

In non-reducing conditions, dimerization occurred at and above 0.21 mg/ml protein concentrations (A). When galectin-13 was dissolved in Laemmli solution containing 10% (v/v) 2-mercaptoethanol, no dimerization of PP13-R was found at all, even at higher protein concentrations (B).



### Placental expressed galectin-13 is phosphorylated

Pro-Q Diamond Phosphoprotein Gel stain - specific for phosphorylated protein side chains - was used to detect previously predicted putative phosphorylation of galectin-13. Both placental purified and bacterially expressed galectin-13 was examined along with ovalbumin (positive control) and BSA (negative control). Strong signal of phosphorylated groups in the lane of ovalbumin and weak signal in the lane of placental-derived galectin-13 could be specifically detected.

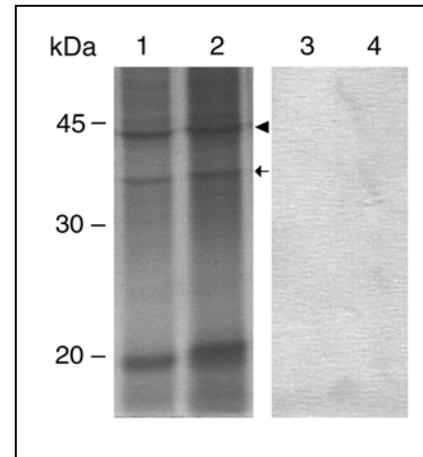


No signal in the lanes of albumin and bacterially expressed galectin-13 was found (A). An equal amount of protein content for each lane was verified by Coomassie staining (B).

### **Galectin-13 binds annexin II and beta/gamma actin**

By Coomassie staining after SDS-PAGE, in cases of isolated and recombinant galectin-13, major bands at 16 or 18 kDa were detected. No additional bands in lower or higher molecular weight regions could be identified, indicating high purity of both protein preparations.

Bands were cut from the gels, then MALDI-TOF MS peptide mapping with MALDI-PSD MS sequencing was performed, recognizing both isolated and recombinant molecule as galectin-13. Next, human term placental tissue and foetal hepatic cell (WRL-68) extracts were bound either to isolated and recombinant galectin-13 coupled to Sepharose 4B, or to Sepharose 4B alone. By Coomassie staining, the same major protein bands at 16



kDa (in case of isolated galectin-13), at 18 kDa (in case of recombinant galectin-13), or at 38 and 41 kDa (in cases of both galectin-13 samples) could be detected either in placental or in foetal hepatic protein extracts bound to either isolated (data not shown) or to recombinant galectin-13 (Fig. 7, Lanes 1-2), while Sepharose 4B did not specifically bind any proteins at all (Fig 7, Lanes 3-4). By MALDI-TOF MS peptide mapping and MALDI-PSD MS sequencing, all protein bands yielded good quality peptide maps, and most of the input masses matched the candidate protein sequences. The eluted 16 or 18 kDa proteins were identified as galectin-13 subunits dimerized with placental derived galectin-13 or recombinant galectin-13 subunits coupled to Sepharose 4B. MALDI-TOF MS data of the 38 kDa protein in both cases permitted the identification of human annexin II (Acc. No.: NM\_004039), while the mass map of the 41 kDa protein matched beta/gamma actin in both cases (Acc. No.: NM\_001101 and NM\_001614). PSD data obtained for precursors also confirmed the identity of these proteins.

### **Poly- and monoclonal galectin-13 antibodies have specific recognition to galectin-13**

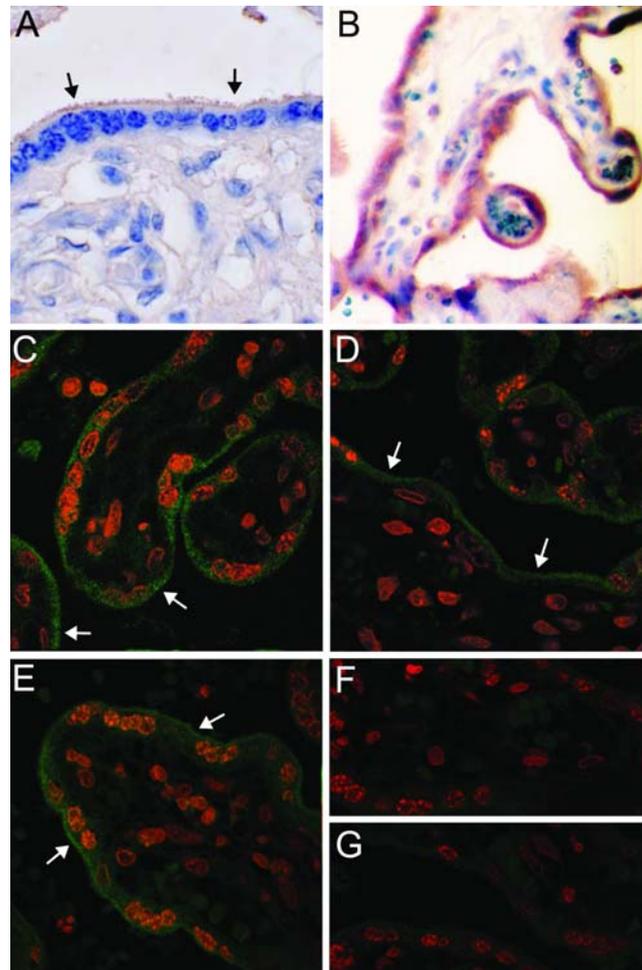
To investigate and compare the specificity of polyclonal and newly developed monoclonal galectin-13 antibodies, Western blot testing was performed utilizing isolated and recombinant galectin-13 and human placental tissue extracts. As previously shown, polyclonal galectin-13 antibody bound specifically to the protein extracted from human term placenta and also reacted with the same size protein in some foetal tissues like liver

and spleen [1]. Here it was observed that polyclonal galectin-13 antibody could recognize the recombinant protein, as it could be observed in cases of isolated galectin-13 and placental expressed galectin-13, with no other proteins recognized. From the newly developed monoclonal galectin-13 antibodies, clone 215 developed against a galectin-13 specific epitope had the strongest reaction with isolated and recombinant galectin-13, and also recognized the placental expressed galectin-13 with no cross-reaction to other proteins of the placenta.

**Galectin-13 is localized predominantly on the brush border membrane of placental syncytiotrophoblasts**

In human term placental tissue, special localization of galectin-13 was found by different immunological techniques. Monoclonal anti-galectin-13 antibody gave a significantly

weaker staining on immunohistochemical sections, while it had stronger staining with confocal imaging than polyclonal galectin-13 antibody. With both antibodies, labelling mainly on the brush border membrane of the syncytiotrophoblasts could be seen by immunohistochemistry, with a parallel weak staining of the cells. By the more sensitive immunofluorescence confocal imaging, a similar, but more intense galectin-13 staining of the brush border membrane was detected, with also a discrete perinuclear labelling of the syncytiotrophoblasts by both



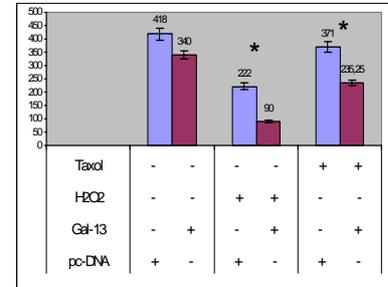
mono- and polyclonal antibodies. Parallel annexin II staining of the syncytiotrophoblasts as well as intense staining on the brush border membrane could be seen. In control sections

stained only with anti-mouse (F) or anti-rabbit (G) secondary IgGs, no staining of the syncytiotrophoblasts or the brush border membrane could be seen.

### Results of cell viability assay

The aim of the test was to prove, whether galectin-13 overexpressing cells are more

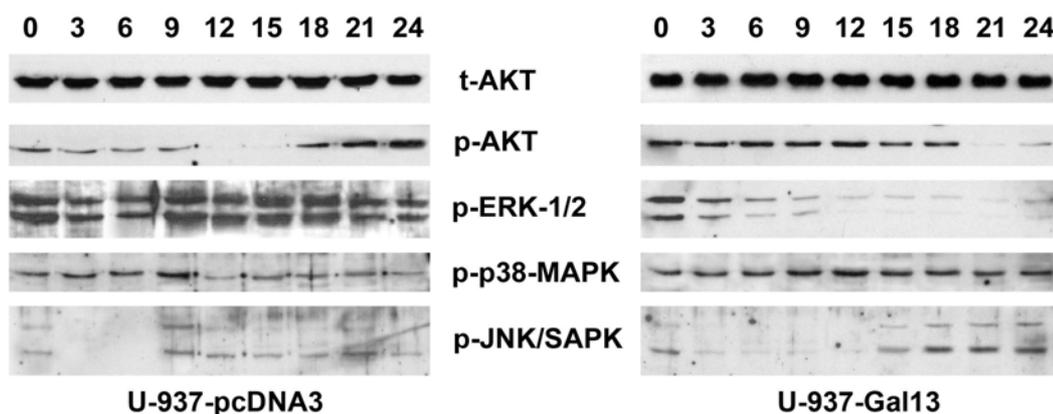
sensible to various stress effects, than the controls are, or it acts as a protective substance in the signal transduction pathways of the cell. Among the applied agents, H<sub>2</sub>O<sub>2</sub> (concentration ≤ 0.3 mM) and Taxol (concentration ≤ 100 nM) were found to have different effect on galectin-13 overexpressing U-937 cells compared to control cells



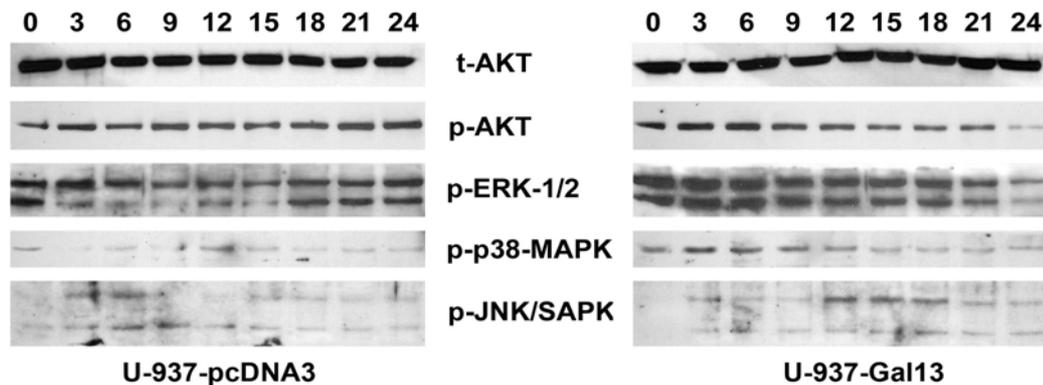
transfected with empty pcDNA3 vector (data not presented). With regard to these results of the MTT assay, following experiments were carried out with these materials.

### Western-blot results of 24 hour H<sub>2</sub>O<sub>2</sub>- and 24 hour Taxol exposition

Treating cells with 100 nM Taxol, a cytostatic agent which blocks the microtubulus formation, the following changes could be observed in cellular signal transduction: till the 18-hour sample, a more remarkable phosphorylation of Akt (p-Akt) could be seen in galectin-13 overexpressing cells, than in controls. After that point of the treatment, phospho-Akt disappeared in galectin-13 containing cells, while it reached its maximum level in control samples. Total Akt level (t-Akt) was constant in all samples. Concerning changes in phospho-Erk-1/2 levels, an increased amount of the protein could be detected in most samples of control cells, in contrast to galectin-13 overexpressing cells. In these latter cases the expression decreased right after the beginning of the experiment and maintained almost undetectable. In cases of Taxol exposition, galectin-13 overexpressing cells showed constant p38-MAPK activation till the end of the experiment. At the same time, decrease of p38-MAPK activation could be observed in control cells from the 12<sup>th</sup> hour. Phosphorylation of JNK/SAPK moderately appeared in galectin-13 overexpressing cells after 15 hours of Taxol exposition.



Treating cells with 0.3 mM H<sub>2</sub>O<sub>2</sub> also resulted in various change in the activation and amount of several signal transduction proteins compared galectin-13 overexpressing cells to simple pcDNA containing control cells. Both galectin-13 overexpressing cells, both control samples were collected in the 0, 3, 6, 9, 12, 15, 18, 21 and 24 hour of the experiment. In cases of galectin-13 overexpression, phospho-Akt was present in the greatest amount in the first three samples, after which a slight decrease could be seen in its level. Finally the active form of the protein totally disappeared. At the same time, in control cells, mild but continuous increase in the level of phospho-Akt could be found from the first hours to the end of the treatment. Total Akt level (t-Akt) was constant in all samples. Concerning Erk-1/2 activation, a very similar pattern could be observed to that of phospho-Akt in galectin-13 overexpressing cells. The most intensive Erk-1/2 activation was present in the 0 – 6 hours of the treatment, after that a prolonged decrease could be followed in its amount in cases of galectin-13 overexpression. At the same time, much lower level of phospho-Erk-1/2 could be seen in the control cells, with a slight increase in its level from the beginning to the end of the treatment. Phospho-p38-MAPK showed modest increase in H<sub>2</sub>O<sub>2</sub> treated galectin-13 overexpressing cells from the first hours. In these cells, JNK/SAPK activation occurred from the 12<sup>th</sup> hour of the experiment. Both p38-MAPK, both JNK/SAPK phosphorylation was lesser in control samples.



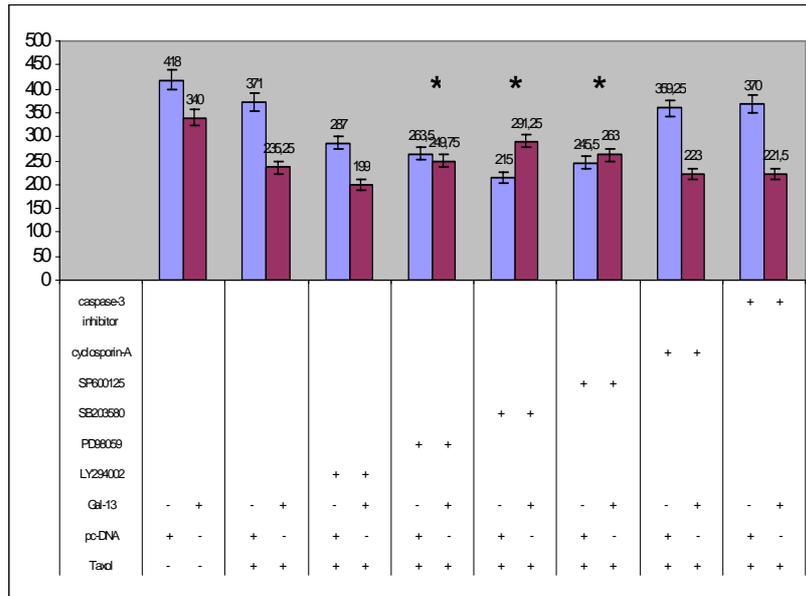
### **Effect of MAPK inhibitors, caspase-3 inhibitor and cyclosporin-A on cell viability**

Results of cell viability assays are presented in tables. It could be observed again, that galectin-13 overexpressing cells had a lower proliferation rate, than control ones. Slight difference in cell survival rate increase between galectin-13 overexpressing cells and controls could be detected in the presence of Erk-1/2-, p38-MAPK-, JNK/SAPK-inhibitors and cyclosporin-A. Concerning the effect of all applied inhibitors, none of them resulted in

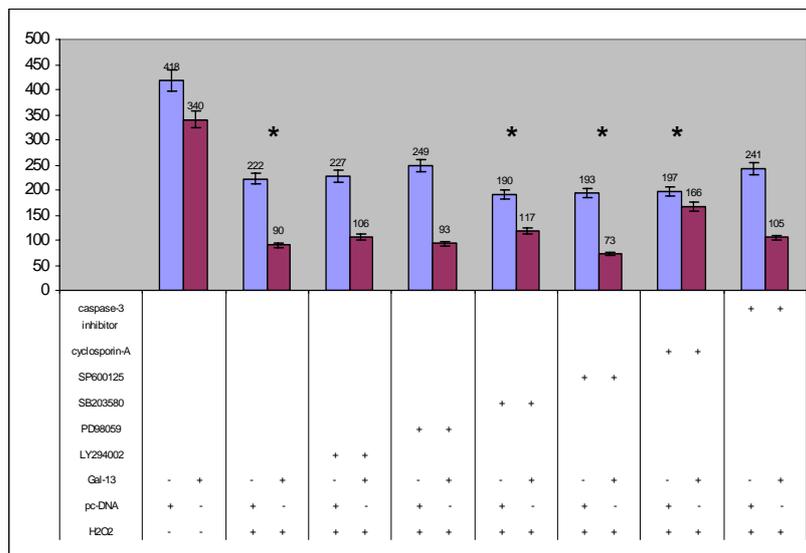
more, than 10 % survival rate increase in galectin-13 overexpressing cells, compared to controls without any further insults (data not shown).

Taxol exposition had significant effect on cell survival rate difference between the two groups when the agent was applied only in 10 nM concentration during 24 hours (1). The treatment resulted in less living cells in galectin-13 overexpressing group, compared to similar treated controls. Although this difference was not as large as it had occurred after H<sub>2</sub>O<sub>2</sub> exposition. Inhibition of p38-MAPK-pathway (SB203580) resulted in the highest increase in cell survival rate of galectin-13 transfectants, but Akt inhibition (LY294002) caused a modestly increased cell death in galectin-13 overexpressing cells, compared to controls. JNK/SAPK inhibition resulted in a modest increase in cell survival of galectin-13 containing cells. Neither cyclosporin-A nor caspase-3 inhibition had any significant protective effect on galectin-13 overexpressing cells in cases of Taxol exposition compared to controls.

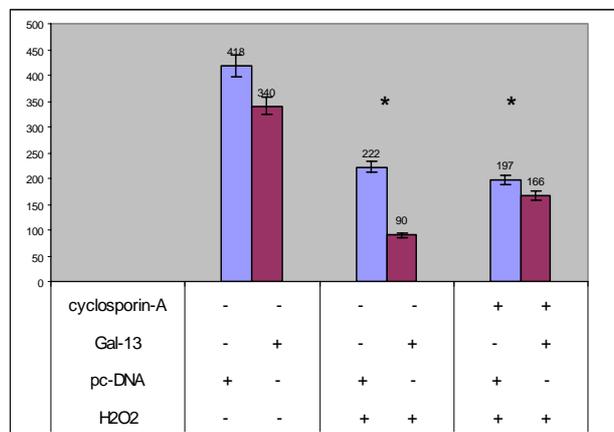
H<sub>2</sub>O<sub>2</sub> exposition brought a considerable difference in cell survival rate between the two kinds of cells (2). The treatment resulted in much less living cells in galectin-13 overexpressing group, compared to similar treated controls. In cases of p38-MAPK inhibition (SB203580), or especially in presence of cyclosporin-A (3), the survival rate of galectin-13 containing cells increased, compared to controls. At the same time JNK/SAPK-inhibition (SP600125) resulted in a modestly decreased survival rate in galectin-13 overexpressing cells. The other applied inhibitors did not result in significant cell survival rate increase or decrease after H<sub>2</sub>O<sub>2</sub> treatment.



1.



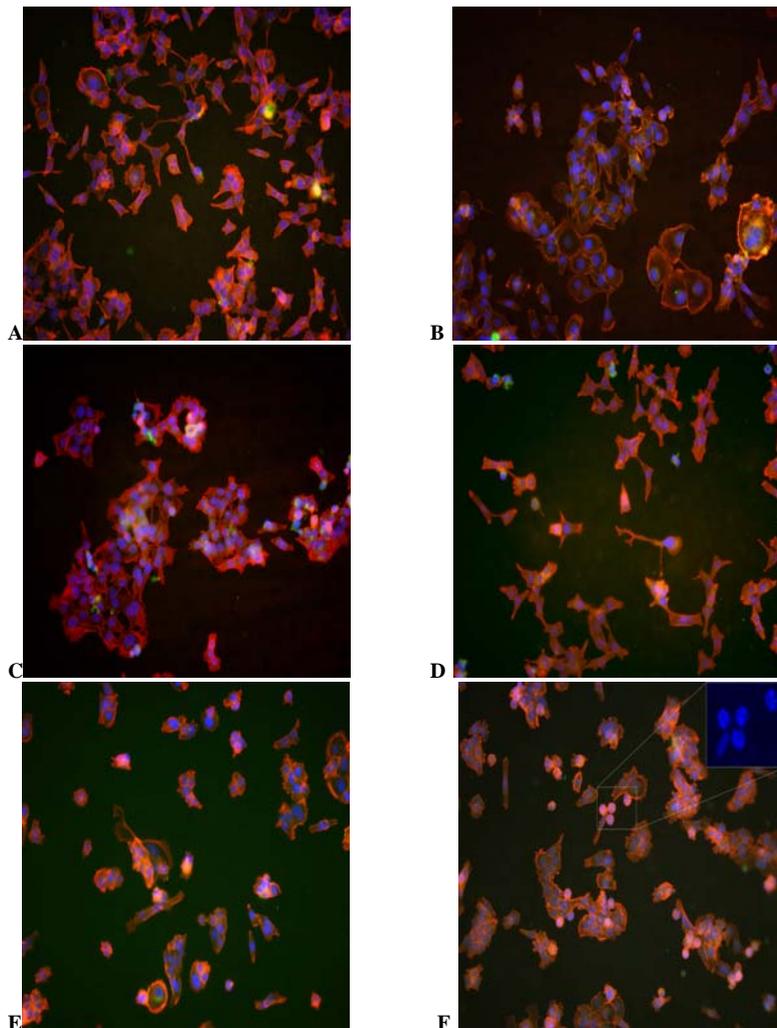
2.



3.

### Results of immunocytochemical studies on cell morphology

In galectin-13 overexpressing U-937 cells a moderate staining of vinculin can be seen in the cell membranes (B) compared to the more intensive labelling of controls' (A). Cells have different shapes, generally swollen, with reduced spikes (B). Control cells show stronger staining of vinculin on their surfaces, and they have normal structures, morphologies, and uniform appearance in their shape (A). After 12 hours of treatment with 0.3 mM H<sub>2</sub>O<sub>2</sub> the spikes of both cell types are reduced or nearly disappeared (C, D). Galectin-13 transfectants are shrunken compared to those of without treatment (D). After Taxol exposition (24 hours, 10 nM), galectin-13 overexpressing cells definitely turn into apoptosis, with shrunken cells, fragmented nuclei (F), while control cells are not effected such dramatically (E).

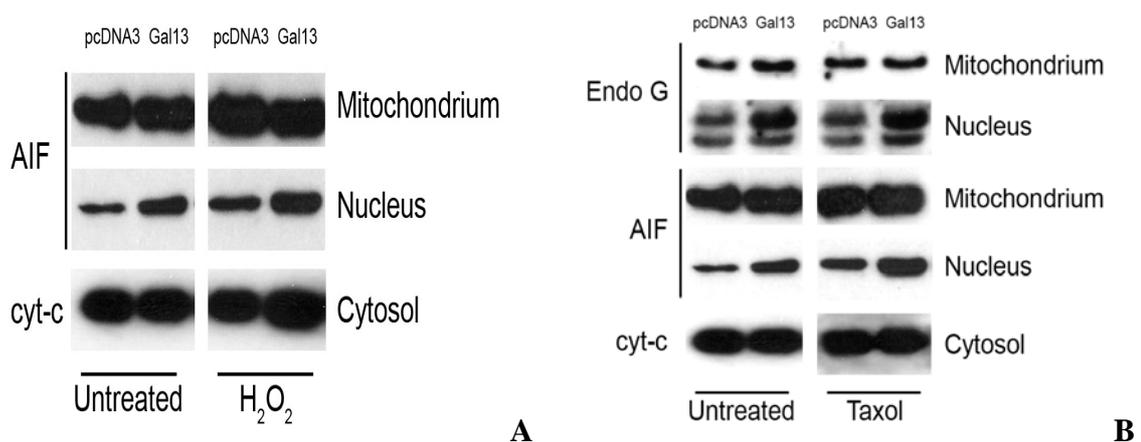


### Detection of mitochondrial protein release and changes in cell signaling pathways

The amount of AIF was found to be the same in mitochondrial fraction of control and galectin-13 overexpressing cells, and did not show any difference after the treatments. At the same time cytosolic AIF content of galectin-13 overexpressing cells was detectable, in contrast to control samples. After treatment with H<sub>2</sub>O<sub>2</sub> (A) or Taxol (B), AIF disappeared from cytosol of galectin-13 containing cells, while it appeared in control samples (data not shown). Nuclear amount of AIF was more in galectin-13 overexpressing cells. After treatments both cell types contained AIF in their nuclei, but more amounts were detectable in galectin-13 overexpressing cells.

Increased nuclear translocation of endonuclease-G from mitochondria was found in galectin-13 overexpressing cells without any further insults and this state was not modified by H<sub>2</sub>O<sub>2</sub> or Taxol exposition (figure is shown only in case of Taxol).

Cytochrom-c release was determined from the cytosolic fraction of the cells. Although, there was no difference between the two cell types in their cytosolic cytochrom-c content without treatment, H<sub>2</sub>O<sub>2</sub> exposition was followed by a greater increase of mitochondrial cytochrom-c release in cases of galectin-13 overexpressing cells, than in controls. This phenomenon was not observed in case of Taxol treatment.

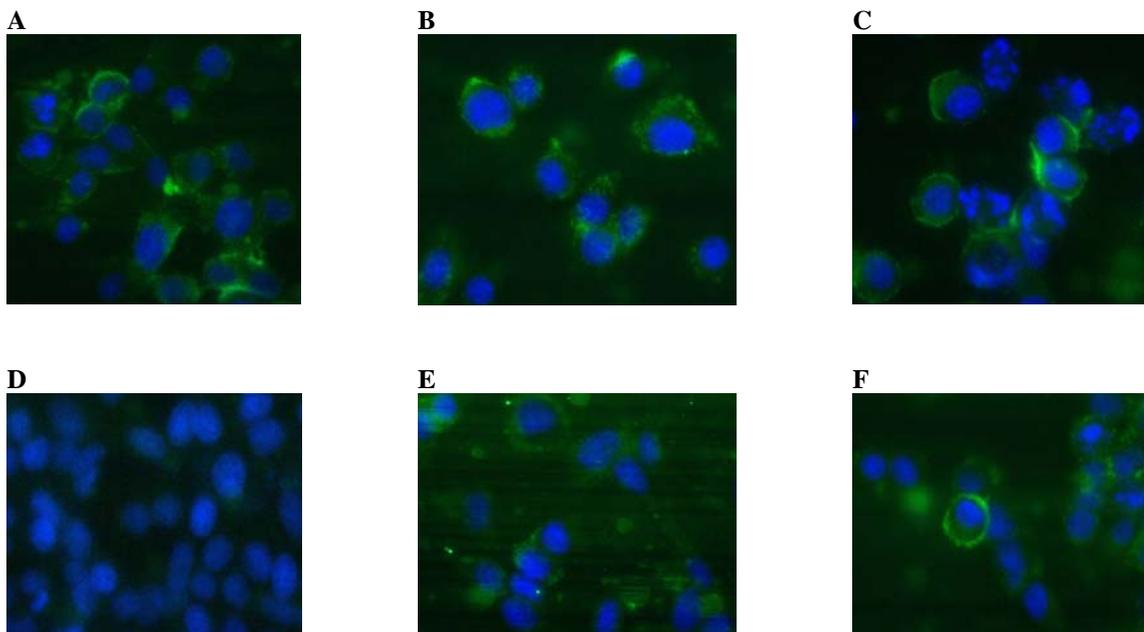


### Measurement of reactive oxygen species (ROS)- production

Galectin-13 overexpressing and control cells had no significant difference between their ROS-producing ability. After 3 hours of 0.3 mM H<sub>2</sub>O<sub>2</sub> exposition, ROS level increased in both cell types, and only hydroxiurea was able to inhibit this process. Other inhibitors of ROS-generating enzymes were not able to abolish H<sub>2</sub>O<sub>2</sub> – induced ROS production. 100 nM Taxol exposition resulted in no changes of ROS-level, compared to cells without any treatment (data not shown).

### Immunocytochemical staining of phospho-Ask-1 proapoptotic protein in pcDNA3/galectin-13 construct or simple pcDNA3 containing U-937 cells

Intensive cytoplasmic phospho-Ask-1 staining can be detected in most galectin-13 overexpressing U-937 cells (B). After 12 hours of 0.3 mM H<sub>2</sub>O<sub>2</sub> exposition phosphorylated Ask-1 appears in all galectin-13 overexpressing cells (D) Performing a 12-hour treatment with 10 nM Taxol, galectin-13 overexpressing cells definitely undergo apoptosis. As the nuclear fragmentation develops, the phospho-Ask-1 staining decreases and disappears (F). Cells transfected with the simple pcDNA3 vector, as controls, did not show any phospho-Ask-1 staining in their cytoplasm before treatment (A). 0.3 mM exposition with H<sub>2</sub>O<sub>2</sub> during 12 hours (C), such as 10 nM Taxol treatment (E), resulted in a positive cytoplasmic staining of control cells as well, but not followed by nuclear fragmentation and absence of phospho-Ask-1 staining, as it could be observed in the progressed stage of apoptosis in galectin-13 overexpressing cells.



### **Detection of cell death by propidium-iodide/FITC-annexin-V staining**

Numerous galectin-13 overexpressing cells were slightly labelled by FITC-annexin-V on their surfaces, while it was lacking in cases of control transfectants. Propidium-iodide staining, however, was present alone in galectin-13 overexpressing cells after H<sub>2</sub>O<sub>2</sub> treatment. Taxol exposition resulted in stronger FITC-annexin-V and PI staining in galectin-13 overexpressing cells than in control transfectants.

### **Measurement of caspase-3 and -7 activity**

Cells without any treatment did not result in notable caspase activation. The same phenomenon was obtained after H<sub>2</sub>O<sub>2</sub> exposition. Following to Taxol treatment, increased caspase activation and consequent fluorescent cytoplasmic labelling was detected in each cell types, but without considerable differences in the labelling of galectin-13 overexpressing and control transfectants (data presented).

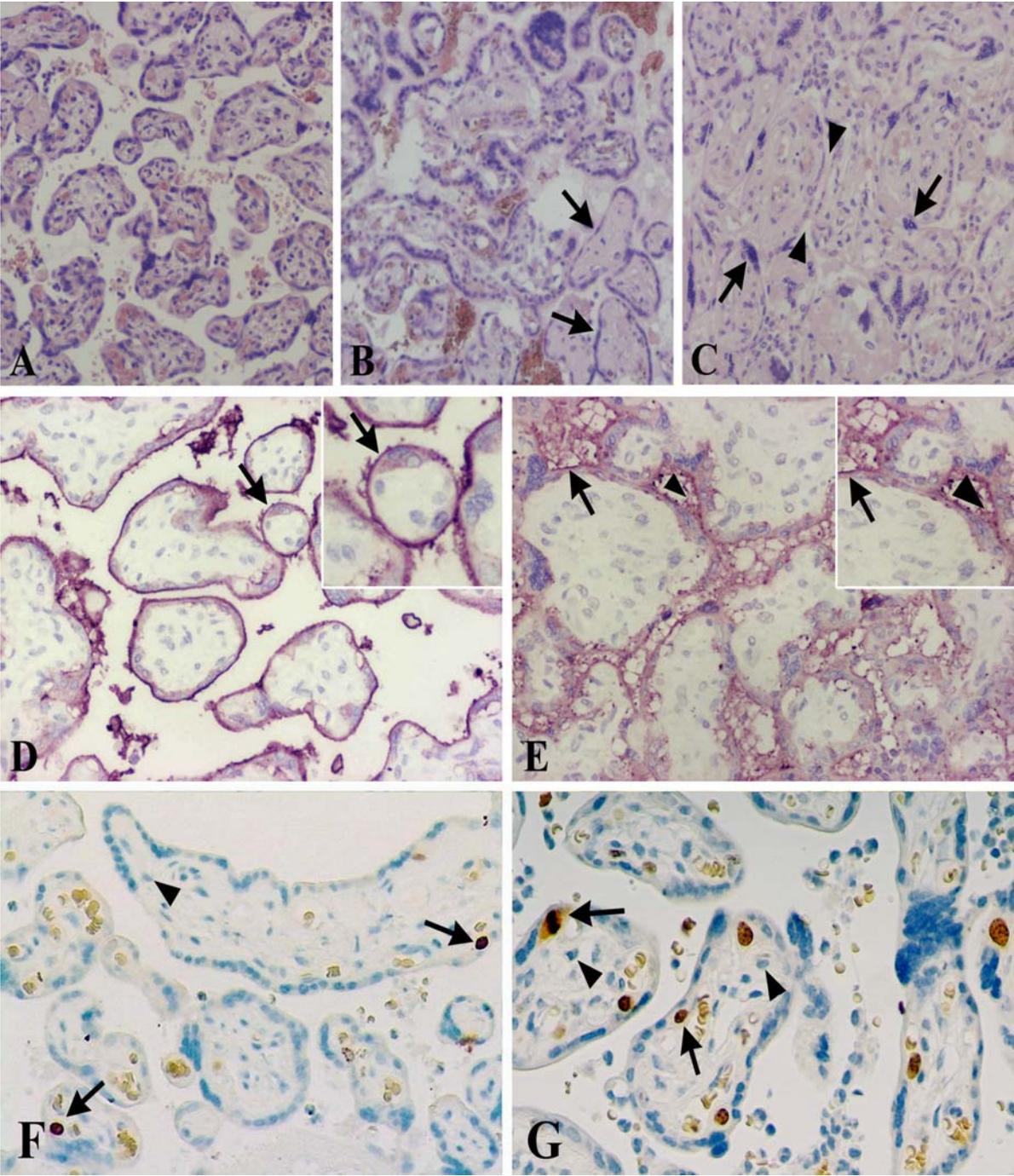
## **Alkaline phosphatase**

### **Histopathology, immunohistochemistry**

In the index case, there were elevated number of syncytial knots on the surface of chorionic villi (**A**). Several groups of avascular tertiary villi with homogenous hyalin-like stroma were also found but fetal vessel thrombosis was not revealed. Presence of “proliferation centres” [65] in immature villi was frequent, unlike in other term placentas (**B**). In several areas, villous crowding were found, where the intervillous space nearly disappeared between the large, poorly vascularised villi (**C**). Histopathology also showed asymmetric intimal fibrin cushions in fetal chorionic vessels. Maternal vessels did not show any specific change. Control placentas showed none of the above listed lesions.

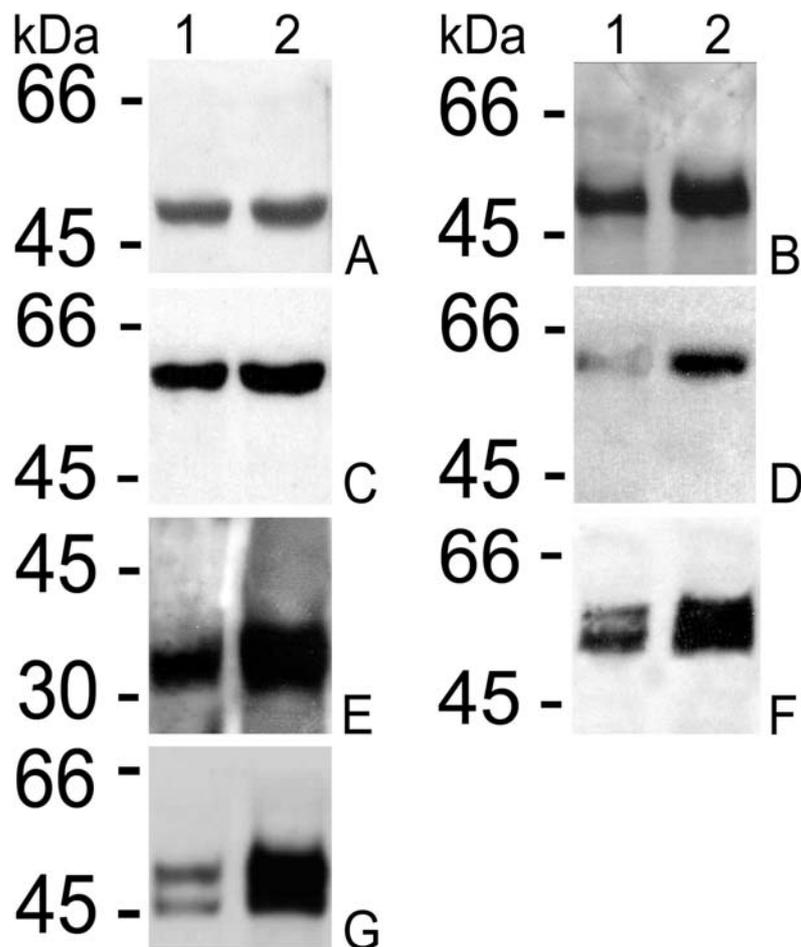
Similarly to literature data [38], in control placentas, AP was normally found on the brush border membranes of the syncytiotrophoblast layer of villus surface (**D**). No staining could be seen in other villous cells like cytotrophoblasts. Staining of the index placenta resulted in a minimal AP labelling of the brush border, and yielded a remarkable diffuse AP positivity in the intervillous space (**E**). Using anti Ki-67 IgG and counting ten fields in every case we examined the average ratio of proliferating cells. In control placentas it was approximately 1-2 % (**F**), whereas a significantly increased positivity of the

cytotrophoblastic cells underlying the syncytium was found in the index placenta with 8-10% of proliferating cells in average (G).



### SDS-PAGE and chemiluminescence Western-blot analysis

In term placental tissue extracts, five different proteins of cellular signal transduction pathways were examined. As shown in, compared to normal placentas, some markers showed remarkable overexpression in the index sample. Phospho-GSK-3 $\beta$  (B) showed a similar increase to that of phospho-Akt (D), whereas differences in total-GSK-3 (A) and total-Akt (C) amounts did not occur. Three other basic signal transduction proteins, phospho-p38-MAPK (E), phospho-SAPK/JNK (F) and phosphorylated p44/42 MAPK / Erk1/2 were found to be overexpressed in the index placenta, compared to controls (G). Densitometric analyses were performed, and the following differences in protein content were found in the index case compared to controls (100%): phospho-GSK-3 $\beta$  152%, total-GSK-3 106%, phospho-Akt 174%, total-Akt 103%, p38MAPK 249%, phospho-p44/42 MAPK / Erk1/2 561% and phospho-SAPK/JNK 202%.



## Discussion

### Galectin-13

Although galectin-13 was first isolated and cloned from human term placenta [14, 1], its expression in human foetal liver and spleen tissues has also been detected [1]. Since galectin-13 showed conserved sequential, structural and computed functional homology to members of the growing  $\beta$ -galactoside-binding galectin family [5], it was designated as galectin-13 [6]. It was verified that galectin-13 mRNA and related ESTs were expressed in placenta, but also in several other tissues and cell types, like fetal liver and spleen, adult brain, eye, mammary gland, skin, testis, cervix, macrophage, breast carcinoma, essential thrombocytaemia, acute myeloblastic leukemia cells, natural killer cell, Kaposi sarcoma-associated herpesvirus infection of primary human dermal endothelial cells, pituitary adenoma subtypes, prostate adenocarcinoma, malignant melanoma cell lines, colon cancer, etc. Galectin-13 gene mapped to the close vicinity of genes of four known and three putative galectins [3, 4, 42-44] with similar exon structures and surrounding untranslated regions in a tight cluster on chromosome-19.

Because of the highly conserved homology with several other galectins, it was likely that galectin-13 exhibited sugar-binding activity. Indeed, in our previous report based on homology modelling [6], the possible functional and structural characteristics of galectin-13 were predicted, including a CRD which resembled the  $\beta$ -galactoside-binding site of galectins. In this study, binding experiments showed that galectin-13 was effectively bound to different sugar containing agarose gels, and that various sugar derivatives could compete this effect with different affinities to the galectin-13 binding site. As in the case of most galectins with similar sugar concentrations applied, N-acetyl-lactosamine had the highest affinity to its CRD. Similarly to CLC protein / galectin-10 but not other previously analyzed galectins, galectin-13 had also high affinity to mannose, which could be understood in terms of the similarities in their CRDs [6, 4]. N-acetyl-galactosamine also had a certain affinity to galectin-13 CRD, in contrast to other sugar derivatives, which only slightly displaced the protein from sugar-coupled agaroses. Interestingly, homology modelling data had also indicated that N-acetyl-lactosamine would bind the most effectively to the galectin-13 binding-site, and in the case of other sugars, there were only minor discrepancies between the previously suggested and experimentally observed

binding affinities [6]. Strong lectin activity of placental-derived and recombinant galectin-13 was also proven by their haemagglutination activity and by haemagglutination inhibition assays, where excess sugar molecules competed with red blood cell sugar residues and proved to be more likely to bind to galectin-13 proteins leaving red blood cells to sediment. These experimental data and the specific and predominant localization of galectin-13 on the brush border membrane of syncytiotrophoblasts were in close agreement with a systematic study on the structure and distribution of specific glycans in human placenta, which showed that residues containing N-acetyl-lactosamine, mannose and N-acetyl-glucosamine were widely expressed on villous surfaces [45]. This may provide an explanation of the binding specificity of galectin-13, and suggests a similar binding pattern of its newly described, mainly placental-expressed homologues.

In vitro, galectin-13 dimerization occurred at and above 0.21 mg/ml concentrations in non-reducing conditions, while in the presence of DTT, no dimerization was detected at all. Furthermore, in reducing conditions, approximately half of the recombinant galectin-13 was bound to sugar-coupled agaroses, and the protein's haemagglutination activity was also abolished, all of which could be explained by the loss of dimerization. Cystein residues in galectins (formerly known as "S-type" or "thiol-dependent" lectins) were considered to be important, because some galectins, such as galectin-1 and galectin-2, but not all, might lose their sugar binding activity under non-reducing conditions [66, 67]. Compared to the structure of CLC / galectin-10 known to be a monomer [68], four additional cystein residues were found in galectin-13, which might localize on a putative dimerization surface. Interestingly, Cys136 and Cys138 on beta-sheet F1 in galectin-13 were found to be missing from all homologues, whereas Cys19 and Cys92 on beta-sheets F2-F3 were missing from homologues including CLC / galectin-10, only the newly described closest relatives contained them. This data may vindicate the surprising experimental findings on its dimerization via disulphide bonds, a phenomenon yet not described for other galectins. Superposing the 3D model of galectin-13 monomer on well known models of galectin dimers [69-71] with RasMol revealed, that if galectin-13 dimerizes like galectin-1 and galectin-2 in a 2-fold rotation perpendicular to the beta-sheets, then only beta-sheets S1 and F1 containing Cys136 might participate in the dimerization. If galectin-13 dimerizes more like to galectin-7, then beta-sheets F1-F5 might comprise the putative dimerization interface containing Cys19, Cys92 and Cys136, as well. As our data is still not enough to

describe an exact dimerization interface, X-ray crystallographic experiments are planning to be started to reveal the proper dimeric structure and carbohydrate binding of the protein.

Our data also showed, that DTT equally decreased the binding strengths between recombinant galectin-13 and different sugars, but no change occurred in the order of their binding capabilities. As the CRD of galectin-13 was situated just opposite the cystein-rich region, it is understandable why the monomeric form could not cross-link red blood cells, but the mechanism of how the reducing agent could decrease the sugar-binding potential of the protein remains to be elucidated. Our sequence alignment also suggested, that the new subfamily members might also dimerize via disulphide bonds, which should be later critically analyzed. As these proteins were primarily expressed in the low blood flow organ placenta, foetal liver and spleen, special alterations in the oxygenization could easily affect their biological functions through their dimerization status and sugar-binding affinities.

It is important to mention that computations localized putative serine and tyrosine kinase phosphorylation sites on the outer surface of galectin-13 at positions 44-52 (Ser48), 37-45 (Tyr41) and 76-84 (Tyr80), in the close vicinity of its CRD. Experimental data showed that in vivo placental expressed and purified galectin-13 was phosphorylated, while the in vitro bacterial expressed protein was not. Knowing that galectin-3 was reported to be phosphorylated at Ser6, and phosphorylation modulated its carbohydrate affinity and biological functions as an “on/off” switch [72, 73], our preliminary data raised the possibility of phosphorylation having an influence on galectin-13’s functional properties. In our conditions, carbohydrate binding affinities were similar both in cases of placental-derived and recombinant galectin-13, the only difference was detected in their lysopholypase activity. Further detailed experiments must be performed to establish the importance and the exact mechanism of this phenomenon.

Earlier it was found that galectin-13 had a weak lysophospholipase (LPL) activity [1], which had previously been observed in the case of CLC protein / galectin-10 [15]. However, it was shown later that this enzymatic activity might not be derived from CLC protein / galectin-10, but from another protein associated to it [4]. Although galectin-13 purified from human term placenta did not contain a similar extent of impurity to what the CLC protein / galectin-10 preparation probably had, we overexpressed the cloned and His-tagged galectin-13 in *E. coli*, and purified it on a Ni-NTA column. Next, the enzymatic activities of placental-derived and recombinant galectin-13 were compared. <sup>31</sup>P NMR analysis showed that both placental-derived and recombinant galectin-13 had a very weak

endogenous LPL activity, even recombinant galectin-13 showed higher catalytic activity than the purified one, indicating that galectin-13 itself possesses LPL activity. Phospholipase (PL) activity of neither the purified nor the recombinant galectin-13 could be confirmed under these circumstances. The exact LPL catalytic centre of galectin-13 must be further analyzed.

Although the possibility that the weak LPL activity of placental-derived galectin-13 coming from an associated and co-purified protein could be excluded, it still remained an interesting question which intracellular proteins were interacting galectin-13. Immobilizing placental-derived and recombinant galectin-13, proteins extracted from term placental tissue and foetal hepatic cells and specifically bound to galectin-13 proteins were determined. By MALDI-TOF MS peptide mapping and sequencing, the 38 kDa and 41 kDa proteins bound both to placental-derived and recombinant galectin-13 were identified as human annexin II and beta/gamma actin, respectively. The 16 or 18 kDa proteins were identified as the eluted placental-derived and recombinant galectin-13 subunits.

It is well known that cells differ widely in their capacity to produce and secrete galectins, and galectin secretion is also responsive to developmental events. During the complex mechanisms of human placentation, correlating with the differentiation pathways of the trophoblasts, changes in the distribution patterns of galectin-1 and galectin-3, homologues of galectin-13, were already seen [74]. Studies also revealed that actin filaments might play an important role in translocation of lectins during differentiation processes [75], and also could be involved in focal concentration of cytosolic galectin at specific cytoskeletal regions, for example at evaginating plasma membrane domains during ectocytosis [76]. The exact mechanisms of how galectin-13 is transported to the outer surface of the syncytiotrophoblasts' plasma membrane has not yet been studied, but it is assumed to be released through an alternative, non-classical route, similarly to other galectins [76]. Fibroblasts were previously shown to secrete galectins by ectocytosis in microvesicles, which also contained actin (derived from disassembling microfilaments) and annexin II in high amounts [76]. As galectin-13 was bound to these molecules very specifically and co-localized with annexin II on the brush border membrane, it possibly utilizes this ectocytotic pathway for externalization, similarly to the secretion of galectin-3 [77]. In vivo, microvesicles were found to be labile and disrupted spontaneously, releasing their lectin cargo very rapidly. Though phospholypases were considered to be candidates for catalyzing the hydrolysis of the sn-2 fatty acyl bond of phospholipids to liberate free

fatty acids and lysophospholipids, and to release soluble extracellular galectins, to date no particular phospholipase has been found to carry out this exact function [78]. Galectin-13 possesses a relatively weak but definitive LPL activity, which is probably insufficient for catabolizing significant amounts of lysophospholipids, but it may contribute to its penetration through the vesicle membrane.

Annexin II, a member of a Ca<sup>2+</sup> and phospholipid binding protein family, is present as a heterotetramere on the apical extracellular surface of the syncytiotrophoblasts [79], co-localized with galectin-13. It is thought to play a role in the differentiation of the placenta and in the functions of the mature microvilli [80]. It was recognized as a profibrinolytic co-receptor for tissue plasminogen activator and plasminogen on endothelial cells, and was shown to stimulate the tissue plasminogen activator-dependent conversion of plasminogen to plasmin [81,82]. In addition, it was also found to promote plasmin inactivation, regulate ion channels, PLA2 and prothrombin activation [83]. Therefore its interaction with galectin-13 may play an important role in placental haemostatic processes.

Though several possible functions of galectin-13 has been suggested based on previous results, it became also our field of interest, whether this protein plays role in cell death processes, like other types of the family, or not.

Based on our Western-blot results, a similar effect on MAPK pathways could be observed in case of H<sub>2</sub>O<sub>2</sub> exposition to that of Taxol treatment, regarding Akt and Erk-1/2-activation. While phosphorylated form of Akt was constantly present or increasing in control cell samples during the 24 hours, it was decreasing and disappeared in galectin-13 overexpressing cells during the experiment. At the same time, total Akt levels did not change in both samples. H<sub>2</sub>O<sub>2</sub> treatment of galectin-13 overexpressing cells resulted in significant elevation of phospho-Erk-1/2, followed by a late decrease and disappearance of it. Control transfectants showed an opposite tendency in Erk-1/2 activation. Several data can be found in literature in the field of galectin-MAPK interactions, as well as in the regards of their pro- and antiapoptotic functions. In connection with galectin-13, clinical data were published in an article with regard to its potential utilization for early serum screening to assess the risk to develop placental insufficiency in the 2nd and 3rd trimesters of pregnancy [84]. The most experiments could be found in connection with galectin-3, a protein that can act either antiapoptotic or proapoptotic role. Overexpression of galectin-3 in J82 human bladder carcinoma cells rendered them resistant to TRAIL-induced apoptosis, whereas phosphatidylinositol 3-kinase (PI3K) inhibitors blocked the galectin-3 protecting

effect. Akt is proven to be a major downstream PI3K target reported to play a role in TRAIL-induced apoptosis. In a study of Oka N. and al., control vector-transfected J82 cells exhibited low level of constitutively active Akt, resulting in sensitivity to TRAIL. On the other hand, J82 cells overexpressing galectin-3 expressed a high level of constitutively active Akt and were resistant to TRAIL. Blockage of TRAIL-induced apoptosis in J82 cells seemed to be mediated by Akt through the inhibition of BID cleavage [31]. Comparing these data with our experiments on Akt level changes, we could also conclude, that activity of Akt continuously decreased in galectin-13 overexpressing cells, whereby they became more sensible to further insults. At the same time, control vector transfectants produce constant or increasing activation in Akt during the experiment, especially in cases of H<sub>2</sub>O<sub>2</sub> exposition. Another possible mechanism for galectin-3's anti-apoptotic activity has been provided by studying mast cells from galectin-3-deficient mice. These cells were more prone to apoptosis than wild-type cells. They were also found to be defective in the expression of c-jun N-terminal kinase (JNK), and to have much reduced c-jun kinase activity. Since JNK is known to be a major regulator of apoptosis, it is possible that galectin-3 exerts its anti-apoptotic activity by regulating the JNK level. However, this mechanism, even if operative in mast cells, cannot be universally applicable to all cell types [13]. Our results seemed to strengthen this finding, concerning that H<sub>2</sub>O<sub>2</sub> treatment was followed by JNK-activation in galectin-13 overexpressing cells, although, Taxol exposition was not. As it is now well accepted, galectin-3 demonstrates anti-apoptotic activity in response to various apoptotic insults on a wide variety of cells. Galectin-3 transfected Jurkat cells (T lymphoma cell) were found to be more resistant to apoptosis induced by anti-Fas antibodies or staurosporine (protein kinase inhibitor) compared to the non-transfected control cells [26]. Human breast carcinoma cells, in which galectin-3 was overexpressed, were more resistant to apoptosis induced by cisplatin, nitric oxide, radiation and anoikis (apoptosis induce by loss of cell anchorage) than controls [27-29]. Contrary to the results obtained in galectin-3 overexpressing cells, galectin-13 made the cells more sensible to cell death following to additional stimuli with several agents, like H<sub>2</sub>O<sub>2</sub> or Taxol.

The molecular mechanisms by which galectin-3 can inhibit apoptosis induced by different stimulation became a field of interest for several research groups. According to data present in publications, several proteins were suggested to be in an interaction with galectin-3, thus mediating its anti- or proapoptotic function. The most featured protein

might be Bcl-2. Galectin-3 was found to share several significant structural properties with this protein. Bcl-2 translocation to the mitochondrial membrane leads to anti-apoptosis activity resulting from blocking cytochrome-c release [85]. It was reported that galectin-3 can also inhibit cytochrome-c release followed by the activation of the caspase cascade when it prevents nitric oxide induced apoptosis in human breast carcinoma BT547 cells [28]. Thus, galectin-3 might be a mitochondrial-associated apoptotic regulator through interaction with Bcl-2 in the cytoplasm. It was also reported that synexin (annexin7), a  $\text{Ca}^{2+}$ - and a phospholipid-binding protein, is required for galectin-3 prevention of mitochondrial damage followed by cytochrome-c release after treatment of cisplatin in BT549 cells. It was also observed that galectin-3 could bind K-Ras and augment its activation [86]. This initiated Ras signal can attenuate ERK but not PI3K activity. K-Ras is a small GTPase that controls complicated networks of apoptotic signaling cascades, so this might be one of the mechanisms of galectin-3 related apoptosis [87].

Different experiences could be found in literature in connection with the possible role of extracellular signal-regulated kinase (Erk) in apoptotic processes. It was observed that only wt-Gal-3 protein coimmunoprecipitated and colocalized with oncogenic K-Ras, resulting in its activation with radical alterations in Ras signaling pathway, whereby the activation of Akt and Ral was suppressed and shifted to the activation of extracellular signal-regulated kinase (Erk). Gal-3-mediated apoptotic resistance and anchorage-independent growth functions could be inhibited by specific inhibitors for Ras or mitogen-activated protein/Erk kinase [88]. Co-transfectants of K-Ras/galectin-3 exhibited enhanced and prolonged epidermal growth factor-stimulated increases in Ras-GTP, Raf-1 activity, and PI3-K activity. Extracellular signal-regulated kinase (Erk) activity, however, was attenuated. Galectin-3 antisense RNA enhanced Erk activation. Thus, unlike galectin-1, which prolongs Ras activation of Erk and inhibits PI3-K, K-Ras-GTP/galectin-3 interactions promote, in addition to PI3-K and Raf-1 activation, a third inhibitory signal that attenuates active Erk [89]. Overexpression of galectin-1 increased membrane-associated Ras, Ras-GTP, and active Erk resulting in cell transformation [90]. The viability of the lymphoid cells found to be reduced by galectin-1 triggered apoptosis, however, the mechanism of the galectin-1 induced apoptosis was still under investigation [24]. Masamune et al. described that galectin-1 activated Erk, JNK, activator protein-1, and NF-kappaB, but not p38-MAPK or Akt. Galectin-1 induced proliferation through Erk, and chemokine production mainly through the activation of NF-kappaB, and in part by JNK and

Erk pathways [91]. According to data of Fischer et al., growth inhibition by galectin-1 occurred in epithelial tumor cell lines from different origins. Antiproliferative effects result from inhibition of the Ras-MEK-Erk pathway and consecutive transcriptional induction of p27 [92]. Galectin-13 overexpressing cells produced a remarkable Erk-1/2 activation from the beginning of the 24 hour treatment with H<sub>2</sub>O<sub>2</sub>, followed by a decrease in its level in the last hours. Erk-1/2 activation increased only during the final period of experiment in control transfectants. Taxol treatment resulted in a continuous attenuation of Erk-1/2 activity in galectin-13 overexpressing cells, while controls showed a constant high level of the active form. Based on our results we concluded, that both Akt, both Erk pathways have an important role in cell death resistance of U-937 cells, since galectin-13 overexpressing variants showed the attenuation of activity both of these kinases during the 24 hours, contrary to control cells. Taxol treatment, however, resulted in an increase in Akt activity and decrease in Erk activity of control samples, a phenomenon, which was described by Elad-Sfadia et al [89]. Thus, these cells did not suffer from apoptotic stimuli, caused by the cytostatic agent, unlike galectin-13 transfectants, which protein promoted decrease in Akt activity and, as a consequence, cell death. Results of kinase inhibition assay also proved the protective effect of either Akt or Erk-1/2 activation in galectin-13 overexpressing cells stimulated by H<sub>2</sub>O<sub>2</sub> or Taxol. Interestingly, in galectin-13 overexpressing cells without any further insults, Erk cascade seemed to mediate susceptibility of cells on proapoptotic processes, as it has already been described by others in connection with proapoptotic galectins [89].

Numerous data could be found in literature about the possible involvement of free radicals and intracellular oxidation/reduction reactions in galectin mediated cell death. During hepatic ischemia/reperfusion galectin-3 inhibits nitrogen free radical-mediated apoptosis, one of the major death pathways. Galectin-3 inhibition of apoptosis involved protection of mitochondrial integrity, inhibition of cytochrome-c release and caspase activation [28]. According to another publication, in which proapoptotic role of galectin-3 was investigated, it was found, that galectin-3 overexpression may alter intracellular oxidation/reduction reactions affecting the metabolism of glutathione and other thiols. In addition, galectin-3 overexpression inactivated Akt by dephosphorylation. Overexpression of constitutively activated Akt protected BT549 (gal-3) cells from TRAIL-induced cytotoxicity. These data suggested that galectin-3-enhanced TRAIL-induced cytotoxicity is mediated through dephosphorylation of Akt, possibly through a redox-dependent process

[32]. With regard to the results of Mazurek et al., genes affected by introduction of wild-type phosphorylated galectin-3 included those involved in oxidative stress, a novel noncaspase lysosomal apoptotic pathway, cell cycle regulation, transcriptional activation, cytoskeleton remodeling, cell adhesion, and tumor invasion [93]. Hahn and al observed that galectin-1 binding to human T cell lines triggered rapid translocation of endonuclease G from mitochondria to nuclei. However, endonuclease G nuclear translocation occurred without cytochrome-c release from mitochondria, without nuclear translocation of apoptosis-inducing factor (AIF), and prior to loss of mitochondrial membrane potential. Galectin-1 treatment did not result in caspase activation, nor was death blocked by caspase inhibitors [94]. Our finding suggested that galectin-13 induced cell death might involve mitochondrial damage, as galectin-13 overexpressing cells reacted on further cell death inducing stimuli with increased cytochrom-c release and nuclear translocation of mitochondrial-derived AIF and endonuclease-G. However, reactive oxygen species (ROS) did not seem to play role in cell death promoting effect of galectin-13. Labelling cells with the Magic Red<sup>TM</sup> Caspase Detection Kit, and regarding that caspase-3 and -7 inhibition did not result in significant cell viability increase, it was also concluded that galectin-13 induced cell death did not involve caspase-3 or -7 effect.

Besides our experiences with galectin-13, additional studies were published about the promoting effect of several galectins on apoptotic agent induced cell death. HeLa cell transfectants overexpressing galectin-7 were found to exhibit a higher rate of apoptosis compared to control transfectants, when exposed to various apoptotic stimuli, including UV-irradiation, actinomycin D, etoposide, camptothecin, and a combination of TNF- $\alpha$  and cycloheximide. The results suggest that galectin-7 acts at a common point of apoptosis-signaling pathways. The following results were obtained with regard to the mechanism: increased cleavage of procaspase-3, which was inhibited by the pan-caspase inhibitor zVAD-fmk, and significantly more cytochrome-c was released in galectin-7 transfectants compared to control cells undergoing apoptosis, suggesting that galectin-7 functions upstream of cytochrome-c release. The zVAD-fmk had no inhibitory effect on the enhanced cytochrome-c release found in galectin-7 transfectants, suggesting that caspases are not involved in this lectin's apoptosis-signaling pathway upstream of cytochrome-c release. Finally, following exposure to the apoptotic stimuli, galectin-7 transfectants exhibited a significant rise in the activity of JNK, an important regulator of apoptosis. DNA microarray comparisons of the gene expression pattern between galectin-7 and control transfectants

have revealed a number of genes whose expression is affected by galectin-7 [34]. These gene products have been linked to regulation of apoptosis, and significantly, are redox-related. Since redox is intimately related to apoptosis, the finding suggests a possibility that galectin-7 might regulate the expression of gene products that modulate the redox status of the cell, resulting in promotion of apoptosis. Concerning our Western-blot results on JNK and p38-MAPK activation, such as cytochrom-c release and AIF or endonuclease-G translocation, it is suggested, that galectin-13, like galectin-7 can act as a proapoptotic protein that functions intracellularly upstream of MAPK activation and cytochrome-c release. As it was observed in cases of galectin-7 overexpression, galectin-13 may operate by affecting the expression of some gene products that are related to the redox status of the cell and are most relevant to the cell death susceptibility of galectin-13 transfectants.

Although, lesser data were presented, but following members of the galectin family (type 1, 9 and 12) have also been shown to induce apoptosis and promote cell death [13].

Galectin-13, as it was published earlier, found to be in an interaction with cytoskeletal elements, like actin [17]. It is not clear now, whether it bears any relation to the results obtained in connection with MAPK pathways in galectin-13 overexpressing cells. Levy et al. found that ligation of integrins by galectin-8 triggers a distinct pattern of cytoskeletal organization, including formation of F-actin-containing microspikes. This is associated with activation of integrin-mediated signaling cascades (ERK and PI3K) They further suggested that downstream effectors of PI3K, including Akt/PKB and p70 S6 kinase, in part mediate cell adhesion, spreading, and microspike formation induced by galectin-8 [11]. It was published by Rawal and al., that one of the *Entamoeba histolytica*'s virulence factors, an adherence lectin (Gal/GalNAc, 260 kDa), activated MAPK cascade, implicating in a variety of physiological cellular functions including apoptosis, proliferation, cytoskeleton rearrangements and permeability changes [95]. Our immunocytochemical results by actin-vinculin-nucleus staining, compared to these observations above, suggested a possible role of galectin-13 in changes of cytoskeletal organization and following activation of the MAPK cascades.

Another immunocytochemical finding was the appearance of phosphorylated apoptosis signal kinase-1 (ASK-1) in the cytoplasm of galectin-13 overexpressing cells, while control transfectants did not show that labelling. H<sub>2</sub>O<sub>2</sub> exposition resulted in the activation of ASK-1 in several control cells as well, while all of the galectin-13 overexpressing transfectants were positive. Taxol treatment, as a well known stimulus of apoptosis, was followed by an

advanced stage of apoptosis in galectin-13 transfectants, since several cells became shrunken, underwent nuclear fragmentation and, at the same time, lost their ASK-1 positivity. These enhanced processes of apoptosis were not seen in control cells. Regarding data in literature, apoptosis signal-regulating kinase-1 is activated in response to various cytotoxic stresses including TNF, Fas and reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub>, and activates JNK and p38MAPK [96]. Apoptosis-associated redox change could also activate mitochondrial permeability transition and apoptosis signaling kinase-1 (ASK-1) [97]. Our experiments could also exactly strengthen these facts, since p38-MAPK, and in a later stage of the 24 hour treatment, JNK/SAPK activation also occurred after H<sub>2</sub>O<sub>2</sub> exposition. Taxol exposition resulted in an increasing activation of p38-MAPK, followed by its attenuation in galectin-13 overexpressing cells, without considerable phosphorylation of JNK/SAPK. Control transfectants produce a slight activation of p38-MAPK, such as JNK/SAPK. Inhibiting p38-MAPK or JNK/SAPK activity with their well known inhibitors (SB203580, SP600125), we could observed that inhibition resulted in the increase in cell viability of galectin-13 transfectants. The same effect was found in case of p38-MAPK inhibition in H<sub>2</sub>O<sub>2</sub>, but mainly Taxol treated galectin-13 overexpressing cells. JNK/SAPK, however, was found to mediate a protective effect in cases of H<sub>2</sub>O<sub>2</sub> exposition, since its inhibition resulted in a decrease in cell viability.

Summarising our experiences with transfected cells, galectin-13 is suggested to have a sensibiliser effect on necrotic/apoptotic susceptibility of transfected U-937 cells. Among the complex and often diverse role of certain MAP kinases, it can be observed that Akt and JNK/SAPK activation acts as pro-survival mediators in galectin-13 overexpressing cells, supplemented with considerable changes in mitochondrial integrity, followed by cytochrom-c release, when they are exposed to H<sub>2</sub>O<sub>2</sub>. On the other hand, ASK-1 phosphorylation and consequent p38-MAPK activation, parallel with nuclear translocation of AIF and endonuclease-G are elements of a proapoptotic way, stimulated by low concentration of Taxol.

Completing our new results with previous experiences, galectin-13 is found to localize in cytoplasm and cell membranes of the transfectants and syncytiotrophoblasts, co-localized with cytoskeletal elements, like actin. Moreover, galectin-13 functions as an intracellularly upstream member of Ask-1 mediated p38-MAPK activation, AIF and endonuclease-G translocation, mitochondrial permeability regulation. Its overexpression results in the susceptibility of cells for additional apoptotic or necrotic insults, thus resulting in cell death after the depletion of protective pathways.

## Alkaline phosphatase

Only a few publications could be found dealing with isolated extreme elevation of AP during pregnancy [98, 99]. Numerous diseases were known to be related to high AP levels [38,39,41]. According to data present in the literature [100], elevated AP level could forecast premature birth in the second trimester, but this parameter is clinically useless because of its low sensitivity and specificity. Placental infarcts or abruption could also be the reason of increased AP levels in the maternal sera. In non-pregnant women with high AP, it is always necessary to exclude any kind of malignancies. Fishman et al. was the first to report on malignancy derived „placental-like alkaline phosphatase” (PLAP) production [39]. Increased levels of PLAP have been found in 15-64% of ovarian cancers, 54% of colorectal carcinomas and 40% of lung cancers [41,101]. PLAP activity was found to be below 1 % of all APs in non-pregnant, non-smoking women, while its connection with numerous malignant diseases has been shown.

Concerning our case, we could exclude liver, kidney, bone and immunological diseases, as well as thyroid dysfunction. Intrauterine growth retardation, preeclampsia and Down syndrome were also excluded. The mother did not smoke or receive any drug treatment. As the level of  $\beta$ -hCG was found to be in the normal range at the time of hospitalisation (in our case 17,706 IU/ml was measured, with a reference range between 94-60,000 IU/ml at the 29-40<sup>th</sup> week of pregnancy) and did not change considerably, syncytiotrophoblastic disorder was originally not suspected. To gain better insight into the morphological and functional changes leading to this phenomenon, several types of pathological and biochemical examinations were carried out. Numerous cellular signal transduction pathways via well established protein-kinases that might lead to proliferation were also investigated.

In this study, histopathological examination found several avascular villi in one block, intimal fibrin cushions in fetal vessels and diffuse syncytial knot formation on villous surfaces. In several areas, proliferation of the cytotrophoblasts was detected and persistence of embryonal villi was present.

Immunohistochemical and Western-blot results also pointed to an increased rate of cytotrophoblast proliferation. Amount of Ki-67 positive proliferating cytotrophoblastic cells were found to be five times more in the index sample than in the controls. A

remarkable elevation of phosphorylated p44/42 MAPK / Erk1/2, known to be a signal transduction factor of proliferation and differentiation, was detected indicating an increased tendency for cell differentiation and syncytiotrophoblast formation. Elevated amounts of other protein kinases, p38-MAPK and SAPK/JNK, also known to take part in signal transduction of cell activation were detected. Elevated amounts of phospho-Akt (active form) and phospho-GSK-3 $\beta$  (inactive form) together with a slight difference between the total-Akt and total-GSK-3 suggested a possible inhibition of the apoptotic pathway. As published previously, Akt is known to regulate cellular processes in response to phosphatidylinositol-3 kinase (PI3K) activation. Phospho-Akt transduces signals to activate cell growth, survival, proliferation and differentiation, while inhibiting proapoptotic signals via phosphorylation and thus inactivation of GSK-3 [102]. Summarizing our observations, we suggest that changes in the expression of phosphorylated p44/42 MAPK / Erk1/2 parallel with the immunohistochemical findings of Ki-67 expression show an intensive cytotrophoblastic proliferation. Apoptosis inhibition is mediated by at least two pathways. Phospho-Akt inactivates GSK-3, a proapoptotic factor, while p38MAPK, a substantial element of cell survival might also indicate the activation of an alternative antiapoptotic process. According to the data in literature, cell activating effect of p38MAPK comes true via several transcription factors (Stat-1, Atf-2, etc.), the same as JNK generates a similar effect by JUN activation [103].

In control placentas, strong AP labelling of the syncytiotrophoblasts' brush border membrane was found, while minimal AP staining of the brush border in immature villi of the index placenta was detected, with a remarkable diffuse AP positivity in the intervillous space. According to the most appropriate hypothesis, loss of the syncytial membranes in immature villi led to increased AP levels in maternal circulation and decreased AP staining of the placenta. Loss of the syncytium might also stimulate increased proliferation of villous cytotrophoblasts, which would then fuse and maintain the syncytium.

Cytotrophoblast proliferation and AP level increase did not affect fetal development in the present case, but draw our attention to its pathophysiology. Our examinations shed new lights on a significant change in placental function, the exact cause of which is still has to be elucidated. In conclusion, when elevated serum AP level is present during pregnancy, differential diagnostically important diseases have to be systematically excluded. We would also recommend precise monitoring of fetal and maternal conditions, histopathological

examinations of the placenta, and more attention to the follow-up of AP levels declining after delivery in such cases.

Summarizing the data provided by the experiments, it can be declared, that both galectin-13, both alkaline phosphatase are characteristic members of syncytiotrophoblastic metabolism. Concerning their common feature, they can be associated with pregnancy disorders, as well as with certain malignant diseases. Their diagnostic value in such cases is still under investigation.

### **Acknowledgements**

The author would like to thank to Balázs Sümegi, PhD, DSci, the leader of “Molecular and cellular biochemistry” doctoral school. This project was notably supported by the works of Szabolcs Bellyei, MD, PhD , Andras Szigeti, MD , Nandor Gabor Than, MD, PhD, Elah Pick and her colleagues. Thanks to György Szekeres, MD, PhD for technical assistance in immunostaining. I am also very grateful for all the participants of these experiments, doctors, PhD students and assistants of Institute of Biochemistry and Medical Chemistry for their help. This work was supported by Hungarian Grants ETT T-09 163/01, 149/2003; FKFP 0166/2001; OMFB-BIO 00201/2002; and OTKA T/020622, T/023076, T/029824, T/046473, M/36996.

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## **Aims of the study**

In this study the possible pathological, pathophysiological role of two syncytiotrophoblast-derived proteins was examined:

Galectin-13 is a member of the growing family of  $\beta$ -galactoside-binding lectins, which have been proved to have diverse biological functions, including regulation of cell adhesion, cell growth, and apoptosis [7-11]. Our aim was to examine these functions and roles of galectin-13.

Alkaline phosphatase (AP) is known to be produced by the liver, bones, small intestine and kidneys, while different AP isoforms are also expressed by the placenta during pregnancy. As the etiology of extreme alkaline phosphatase elevation in pregnancy is still unknown and no literature data could be found dealing with its cellular background, we decided to examine the biochemical and pathophysiological events of this phenomenon.

We planed the following experiments in order to get answer to our questions:

1. Comparison of galectin-13 cDNA and amino acid sequences to various EST, genomic and protein databases by BLAST at NCBI, 3D modelling of galectin-13.
2. Galectin-13 lysophospholipase (LPL) activity detection by NMR.
3. Galectin-13 sugar-binding ability.
4. Galectin-13 haemagglutination assay.
5. Galectin-13 dimerization assay.
6. Pro-Q Diamond Phosphoprotein Gel staining.
7. Identification of galectin-13-bound proteins by mass spectrometry.
8. Localization of galectin-13 in syncytiotrophoblasts by immunofluorescence confocal microscopy.

9. Examination of the effect of H<sub>2</sub>O<sub>2</sub>- and Taxol-exposition on galectin-13 overexpressing cells. Analysis of MAPK-phosphorylation by Western blot, determination of MAPK-activation profile.
10. Studies on the effect of MAPK inhibitors', caspase-3 inhibitor and cyclosporin-A on cell viability.
11. Immunocytochemical studies on cell morphology.
12. Detection of mitochondrial protein release and changes in cell signalling pathways.
13. Measurement of reactive oxygen species (ROS) - production.
14. Immunocytochemical detection of Ask-1 phosphorylation/activation in apoptosis.
15. Detection of cell death by propidium-iodide/FITC-annexin-V staining.
16. Measurement of caspase-3 and -7 activity.
17. Histopathology and immunohistochemistry of normal and elevated HSAP-level associated placental tissues.
18. Protein expression studies on normal and elevated HSAP-level associated placental tissues.

## General conclusions

1. By GenBank search of related EST sequences, it could be assumed that galectin-13 and its EST sequence appears in numerous fetal and adult tissues, and gene expression array-based results also suggested the presence of galectin-13 transcripts in several normal cells or malignancies (NCBI database; Geo Profiles) as well. Galectin-13 gene mapped to chromosome 19 (19q13.1) in the close vicinity of genes of four known (galectin-10, galectin-7, galectin-4 and placental protein 13-like protein) and three putative galectins at 19q13.1-13.2 with similar exon structures, indicating their common genetic origin. Computational 3D modelling based on its primary structure and homology to prototype galectins revealed a characteristic “jellyroll” fold (deposited to Brookhaven Data Bank, Acc. No.: 1F87).
2. Galectin-13 possesses weak endogenous LPL activity. For both isolated and recombinant galectin-13, the highest degree of transformation was found for L- $\alpha$ -lysophosphatidylcholine (1-acyl-glycero-3-phosphorylcholine, LPC) according to the  $^{31}\text{P}$  NMR spectra.
3. Non-modified agarose beads (Sephadex 2B) did not bind galectin-13 at all, while all types of sugar-coupled agarose beads bound more than 95% of galectin-13 after 1 h incubation. Different sugars (1mM – 1 M) eluted the protein from various sugar-coupled agarose in different manners, with the following elution capacity: N-acetyl-lactosamine > mannose > N-acetyl-galactosamine > maltose > glucose > galactose > fucose > lactose. In 1 M concentration, N-acetyl-lactosamine had significantly the highest efficacy (95-100%) to elute galectin-13 from all kinds of beads.
4. In non-reducing conditions, very small amounts of galectin-13 induced haemagglutination, and strong agglutination was detected at and above 50  $\mu\text{g/ml}$  applied protein concentrations, which was very similar to the phenomenon seen in cases of other galectins.
5. In non-reducing conditions, dimerization occurred at and above 0.21 mg/ml protein concentrations. When galectin-13 was dissolved in Laemmli solution containing 10 %

(v/v) 2-mercaptoethanol, no dimerization of galectin-13 was found at all, even at higher protein concentrations.

6. Strong signal of phosphorylated groups in the lane of ovalbumin (positive control) and weak signal in the lane of placental-derived galectin-13 could be specifically detected. No signal in the lanes of albumin (negative control) and bacterially expressed galectin-13 was found.
7. 38 and 41 kDa proteins could be detected either in placental or in foetal hepatic protein extracts bound to either isolated or to recombinant galectin-13. MALDI-TOF MS data of the 38 kDa protein in both cases permitted the identification of human annexin II (Acc. No.: NM\_004039), while the mass map of the 41 kDa protein matched beta/gamma actin in both cases (Acc. No.: NM\_001101 and NM\_001614).
8. By immunofluorescence confocal imaging, intensive galectin-13 staining of the brush border membrane was detected in syncytiotrophoblasts, with also a discrete perinuclear labelling of the cells by both mono- and polyclonal antibodies.
9. Treating cells with 100 nM Taxol, a cytostatic agent which blocks the microtubulus formation, phospho-Akt and phospho-Erk-1/2 decreased and disappeared in galectin-13 containing cells, while it reached its maximum level in control samples. Total Akt level was constant in all samples. Galectin-13 overexpressing cells showed constant p38-MAPK activation till the end of the experiment. At the same time, decrease of p38-MAPK activation could be observed in control cells. Phosphorylation of JNK/SAPK moderately appeared in galectin-13 overexpressing cells. Treating cells with 0.3 mM H<sub>2</sub>O<sub>2</sub> also resulted in decrease in phospho-Akt level and Erk-1/2 activation of galectin-13 overexpressing cells. Total Akt level was constant in all samples. Phospho-p38-MAPK showed modest increase in H<sub>2</sub>O<sub>2</sub> treated galectin-13 overexpressing cells from the first hours. In these cells, JNK/SAPK activation occurred from the 12<sup>th</sup> hour of the experiment. Both p38-MAPK, both JNK/SAPK phosphorylation was lesser in control samples.
10. Inhibition of p38-MAPK-pathway (SB203580) resulted in the highest increase in cell survival rate of galectin-13 transfectants after low dose Taxol treatment. JNK/SAPK

inhibition resulted in a modest increase in cell survival of galectin-13 containing cells. Neither cyclosporin-A nor caspase-3 inhibition had any significant protective effect on galectin-13 overexpressing cells in cases of Taxol exposition compared to controls. H<sub>2</sub>O<sub>2</sub> exposition resulted in much less living cells in galectin-13 overexpressing group, compared to similar treated controls. In cases of p38-MAPK inhibition (SB203580), or especially in presence of cyclosporin-A, the survival rate of galectin-13 containing cells increased, compared to controls. At the same time JNK/SAPK-inhibition (SP600125) resulted in a modestly decreased survival rate in galectin-13 overexpressing cells. The other applied inhibitors did not result in significant cell survival rate increase or decrease after H<sub>2</sub>O<sub>2</sub> treatment.

11. After 12 hours of treatment with 0.3 mM H<sub>2</sub>O<sub>2</sub> the spikes of both cell types are reduced or nearly disappeared. Galectin-13 transfectants are shrunken compared to those of without treatment. After Taxol exposition, galectin-13 overexpressing cells definitely turn into apoptosis, with shrunken cells, fragmented nuclei, while control cells are not effected such dramatically.
12. Increased nuclear translocation of endonuclease-G from mitochondria was found in galectin-13 overexpressing cells without any further insults and this state was not modified by H<sub>2</sub>O<sub>2</sub> or Taxol exposition. H<sub>2</sub>O<sub>2</sub> exposition was followed by a greater increase of mitochondrial cytochrom-c release in cases of galectin-13 overexpressing cells, than in controls. This phenomenon was not observed in case of Taxol treatment. Nuclear amount of AIF was more in galectin-13 overexpressing cells. After treatments both cell types contained AIF in their nuclei, but more amounts were detectable in galectin-13 overexpressing cells.
13. Galectin-13 overexpressing and control cells had no considerable difference between their ROS-producing ability.
14. Intensive cytoplasmic phospho-Ask-1 staining can be detected in most galectin-13 overexpressing U-937 cells contrary to controls. After 12 hours of 0.3 mM H<sub>2</sub>O<sub>2</sub> exposition phosphorylated Ask-1 appears in all galectin-13 overexpressing cells. Performing a 12-hour treatment with 10 nM Taxol, galectin-13 overexpressing cells definitely undergo apoptosis.

15. Numerous galectin-13 overexpressing cells were slightly labelled by FITC-annexin-V on their surfaces, while it was lacking in cases of control transfectants. Propidium-iodide staining, however, was present alone in galectin-13 overexpressing cells after H<sub>2</sub>O<sub>2</sub> treatment. Taxol exposition resulted in stronger FITC-annexin-V and PI staining in galectin-13 overexpressing cells than in control transfectants.
16. Cells without any treatment did not result in notable caspase activation. The same phenomenon was obtained after H<sub>2</sub>O<sub>2</sub> exposition. Following to Taxol treatment, increased caspase activation was detected in each cell types, but without considerable differences in the labelling of galectin-13 overexpressing and control transfectants.
17. Elevated number of syncytial knots on the surface of chorionic villi, several groups of avascular tertiary villi, presence of “proliferation centres” in immature villi, villous crowding and nearly disappeared intervillous space were found in elevated HSAP-associated placental tissue samples. Staining of the index placenta resulted in a minimal HSAP labelling of the brush border, and yielded a remarkable diffuse HSAP positivity in the intervillous space. 10 % of cells were Ki-67 positive in elevated HSAP-associated samples, compared to 1-2 % positivity of controls.
18. In term placental tissue extracts, five different proteins of cellular signal transduction pathways were examined. Densitometric analyses were performed on Western-blot bands, and the following differences in protein content were found in the index case compared to controls (100%): phospho-GSK-3 $\beta$  152%, total-GSK-3 106%, phospho-Akt 174%, total-Akt 103%, phospho-p38MAPK 249%, phospho-p44/42 MAPK / Erk1/2 561% and phospho-SAPK/JNK 202%.