Effects of overexpression of syndecan-1 in mesenchymal tumor cells.

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A master's thesis in Molecular cell biology

OVEREXPRESSION OF SYNDECAN-1 IN MESENCHYMAL TUMOR CELLS

By

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ABSTRACT

Background
All cells carry a transmembrane proteoglycan called syndecan. Syndecans influence many functions like cell migration, cell adhesion and cell proliferation and it is involved in cellular signaling and tumourigenesis.
The common features of differentiation in two mesenchymal tumor cell types, malignant mesothelioma cells and fibrosarcoma cells, are connected to the synthesis of syndecans. By studying the overexpression of syndecan-1 we hope to discover new features of the syndecan-1 molecule that we can add to the puzzle of mesenchymal tumors.

Methods and findings
Malignant mesothelioma cells and fibrosarcoma cells were cultured and transfected with full-length- and truncated syndecan-1 constructs. To detect the expression of syndecan-1 on RNA level Rt-Q-PCR was conducted followed by immunocytochemical analysis to establish the syndecan-1 expression on protein level. The result showed a 2-7 fold increase of syndecan-1 in the transfectants comparing to the control. The proliferation of transfectants was analyzed by cell proliferation assay and cell cycle analysis. All transfectants showed a lower proliferation rate comparing to the controls and a slight increase in G0/G1 phase.

Because of the high structural similarities of syndecan family members, I studied how overexpression of syndecan-1 affected the other syndecans using Rt-Q-PCR. Syndecan-2 and -4 were downregulated in the transfectants carrying syndecan-1 ectodomain, whereas the truncated versions had the opposite effect. The expression of syndecan-bound heparan sulfate was studied by FACS and indicated an upregulation for heparan sulfate when measuring internal- and membrane bound syndecans simultanesly.

Conclusions
In this study I have shown that overexpression of full-length syndecan-1 and the different truncated variants, had similar profound effects on mesenchymal cell proliferation. Syndecan-1 also influences the other members of the syndecan family suggesting a complex regulation.

BACKGROUND

Syndecans

One group of proteins that participate in the regulation of the mesothelial cell differentiation is the syndecans (sdc). They form a four-member protein family: syndecan -1, -2, -3 and -4. Vertebrates have all four syndecan genes whereas invertebrates only have one. Syndecans are cell surface associated proteoglycans which are involved in cell migration and motility, cell adhesion and cell proliferation of various tumor types[1]. They all contain an intracellular domain, a transmembrane domain and an extracellular domain.
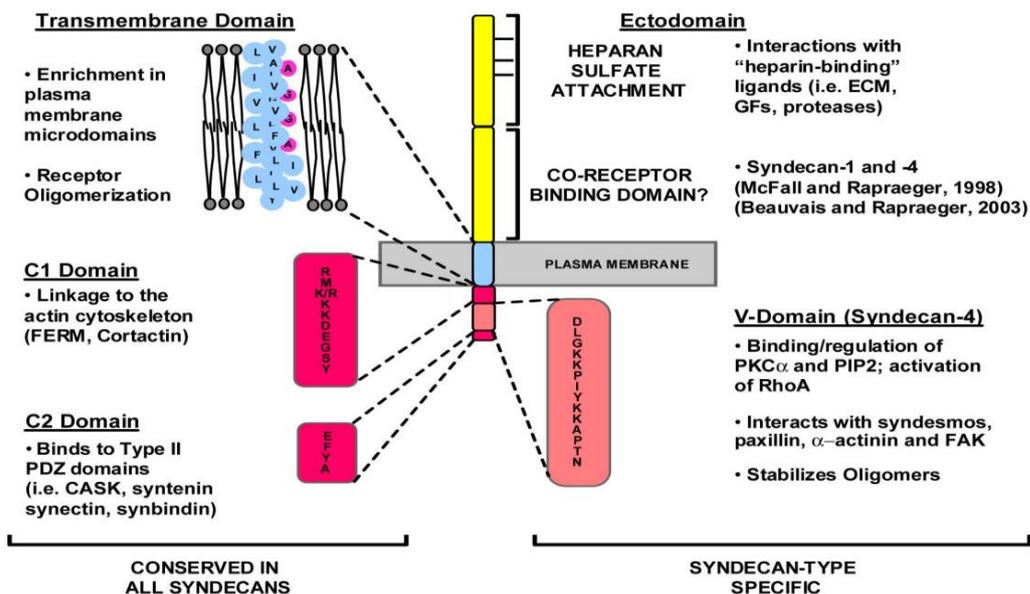

Figure 1.
Schematic illustration of the syndecan-1 molecule containing the ectodomain with the heparan sulfate attachment sites, the endodomain with the conserved (C1, C2) and variable (V) regions. (Picture taken from RB & E homepage www.rbej.com)

The intracellular domain of the syndecans consists of a short cytoplasmic tail that contains two conserved regions C1 and C2 separated by a variable region (V-region). The C-region enables the syndecan family members to form homo-aggregates. C1 is located closest to the cell membrane and is involved in syndecan dimerization and the translocation of syndecan to the cell surface. It also binds to some intracellular proteins like actin microfilaments on the cytoskeleton and contains the RMKKK nuclear localisation signal (NLS). The RMKKK sequence guides the syndecan-1 molecule to the nucleus where it accumulates in a time dependent manner[2]. This short sequence of positively charged amino acids consisting of lysine (K) and arginine (R) has capacity to interact with nuclear structures[3, 4, 5, 6]. In recent studies on mesenchymal tumor cell lines the RMKKK has been proven to decrease the proliferation of the cells[7, 8].

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The C2 region functions as a binding site for proteins like syntenin in the postsynaptic density domain (PDZ) and helps syndecans to bind cytoskeletal proteins and cytosolic signalling molecules. The V-region that separates the two C-regions is syndecan type specific and it gives each of the four syndecans special property in binding to certain proteins. It also has an important role in actin bundling, cell migration and lamellipodial spreading[9].

Between the cytoplasmic tail and the ectodomain is the transmembrane domain which also is highly conserved. It has a classic type 1 membrane protein structure where the syndecan molecule crosses the bilayer as an α helix. The transmembrane portion has the ability to be self-associating[4, 10, 5, 6, 11].

The extracellular domain (ectodomain) has two glycosaminoglycan (GAG) binding regions. Heparan sulfate (HS) is synthesized on the outer of these regions and chondroitin sulfate (CS) on the inner one. These GAGs consist of repeating disaccharide units with variable sulfation on free hydroxyl and amino groups, and regions with specific sulfation patterns enable the GAGs to bind certain proteins. Thus, the HS chains are responsible for the binding of growth factors and various components in the extracellular matrix like collagen, fibronectin and laminin[4, 5].

HS binds not only to FGF-2 but also to its tyrosine kinase receptor FGFR-1. This binding is necessary for the receptor-factor complex to trigger the cascade of intracellular signaling. In this way syndecan-1 may affect proliferation, differentiation and migration of the cell depending on its co-receptor function. It is also possible that these proteoglycans may act as independent receptors[12].

Due to their many functions the syndecans are strictly regulated and they are differently expressed by different cells. Syndecan-1 is mostly synthesized on epithelial cells while syndecans-2 and -4 are more prominent in mesenchymal tissues. Syndecan-3 is typically found in neural tissues (neurocan) but it is also expressed in cartilage. The mesothelium expresses syndecans-1, -2 and -4, while syndecan -3 sequences only give a minute reactivity on Rt-Q-PCR. The epitheloid mesothelioma cells express more syndecans than the others, the difference being most prominent for syndecans -2 and -4. Downregulation of syndecan synthesis in mesothelioma cells also gives a more sarcomatoid growth pattern.

All these events are supposedly due to binding reactions on the cell surface. However, syndecan-1 is also present in the nucleus of the cells. This transport to the nucleus is mediated by tubulin and there may be an association between nuclear syndecan-1 and proliferation. It has been suggested that this internalized syndecan should act as a transport vehicle for a ligand, presumably a growth factor. FGF-2 has also been demonstrated inside the nucleus, and it co-localizes with syndecan-1[6, 12,7].

The role of syndecan-1 in malignant cells and its association with differentiation and proliferation makes this molecule an interesting object in tumor biology and a possible target for future chemotherapy[4, 10, 5, 6].

The mesothelium and mesenchymal tumors

Mesothelial differentiation

In the beginning of embryogenesis, cleavage and gastrulation is an embryo constellation of three germ layers: ectoderm, mesoderm endoderm, which correlates to the outer, middle and respectively inner layers. In later embryonic development, the body cavity (the coelom) is formed by the space between the dorsal and ventral layers of the lateral plate mesoderm. The two layers will later fuse and divide the coelom into the pleural, pericardial and peritoneal cavities. The cells on the surface flatten out and differentiate to an epithelioid morphology. The epitheloid cells are tightly packed and connected by tight junctions and operate like a whole unit. Such cells rest on the basal lamina where they form a protective and non-adhesive monolayer[13, 3].

A basement membrane separates the epitheloid cells from the underlying fibroblast-like cells. These cells are more elongated and imbedded in the matrix. The submesothelial fibroblasts are multipotent, i.e., they have the ability to differentiate into other phenotypes, including to epithelioid cells[3]. Also the epitheloid cells on the surface have the capacity to transdifferentiate into the other growth pattern.

If the mesothelium is injured the healing process depends on whether the injury has affected the basal lamina or not. An injury that leaves the basement membrane intact is easily repaired by neighbouring epitheloid cells that either migrate from the vicinity or attach after having been submersed in the cavity fluid[3]. If the injury is deeper and has affected the basal lamina, the multipontent fibroblast-like cells migrate to the location of injury were they differentiate to a surface cell of epitheloid type.

The mesothelial cells secrete many different molecules, one of them is hyaluronan. Hyaluronan is a glycosaminoglycan (GAG) which is synthesized on the cell membrane where it serves to lubricate the serosal surface. Here it forms the cell microenvironment, and it thereby affects various interactions with the surroundings, which also may influence the possibilities for tumor cells to spread and attach to the pleural surface[14].

Malignant mesothelioma

Malignant mesothelioma (MM) is an aggressive tumor developed in the pleura and it is associated with exposure to asbestos. The long and thin fibers of the asbestos crystal can cause a chronic inflammatory condition (asbestosis), and it may also interfere with the mitosis of the cells. A mechanical disturbance of the mitotic spindle may result in an uneven distribution of chromosomes during the anaphase. Such an event may in turn result in a daughter cell with a growth advantage, i.e., a tumor development has been initiated. The cell may lack suppressor gene functions which in turn, abrogate the genetic stability of the cell.

A second effect of the asbestos crystal may be the generation of reactive oxygen species (ROS) on the crystal surface. These ROS entities may cause DNA damage and further promote the carcinogenic process[14, 15].

The malignant mesothelial cells have often retained the ability of their benign precursors to transdifferentiate. Thus, most tumors simultaneously contain sarcomatoid (fibroblast-like) cells and epithelioid cells which characterize the biphasic mesothelioma. Purely epitheloid or sarcomatoid mesotheliomas are less common. Gene expression studies indicate that the sarcomatoid cells are less mature (less differentiated), which is in line with the observation that patients with dominating sarcomatous components are less responsive to treatment which shortens survival times.

This carcinogenic process is very long, and tumors normally do not appear until 20-50 years after the first exposure to asbestos. The prognosis of this type of tumor is in most cases very poor, with a typical survival time between 4 and 12 months after diagnosis (*for review see* [6]). A small proportion of tumors with dominantly epithelioid components are markedly less aggressive, and some of the mesothelioma patients may live for a decade after diagnosis also without treatment. In general mesotheliomas are highly resistant to most forms of therapies, including chemotherapy, immunotherapy and radiotherapy[14,15].

Fibrosarcoma

Fibrosarcoma is a highly malignant tumor derived from a family of connective tissue cells, the fibroblasts. The fibroblasts secrete extracellular matrix components like collagen I and II and are present throughout the body. In common with the mesothelial cells, the fibroblasts are also multipontent and have the ability to transform into other growth pattern, but lose their differentiation properties along with maturation[3]. The invasion is primarily local and metastases are subsequently formed in the lungs which are followed by a low survival rate. Very little is known regarding the importance of syndecan-1 in fibrosarcoma and hopefully this will open up for future studies.

AIM

The aim of this study is to elucidate the effects of syndecan-1 on cell proliferation. Using different syndecan-1 constructs we can study the importance of the different parts of the molecule as well as whether the effects are initiated in the nucleus or at the cell membrane.

MATERIALS AND METHODS

Cell lines and cell culture conditions

Two well-characerised cell lines were used in this study, the human malignant mesothelioma STAV cell line and human fibrosarcoma B6FS cell line. They are both of mesenchymal origin and have a low endogenous expression of syndecan-1 which makes them suitable for this type of study[13, 16].

The STAV cell line consists of two sub-lines STAV-AB and STAV-FCS, respectively. The STAV-AB cells were grown in RPMI 1640 medium [*Qiagen, Hilden, Germany*] with 25 mM HEPES (hydroxyl-ethyl-piperazine-ethan-sulfonacid) [*GIBCO, Grand Island, NY, USA*] and 5 mM L-Glutamine. The medium was supplemented with 10% human AB serum which allowed the cells to grow with an epitheloid phenotype. Correspondingly, the STAV-FCS sub-line was grown in a mixture of Calf Serum and Foetal Bovine Serum (ratio 1:1) [*Invitrogen, Karlsruhe, Germany*], which resulted in a sarcomatoid growth pattern. The B6FS cell line was grown in RPMI 1640+ GlutaMAX TM -1 [*GIBCO*] supplemented with 10% Foetal Bovine Serum.

All cell lines were cultured in 75 cm^3 tissue flasks in 37° C in 5% CO_2 [17]. The medium was changed every 2-3 days and cells were split at confluence by using 5% Trypsin-EDTA [*GIBCO Introgen*].
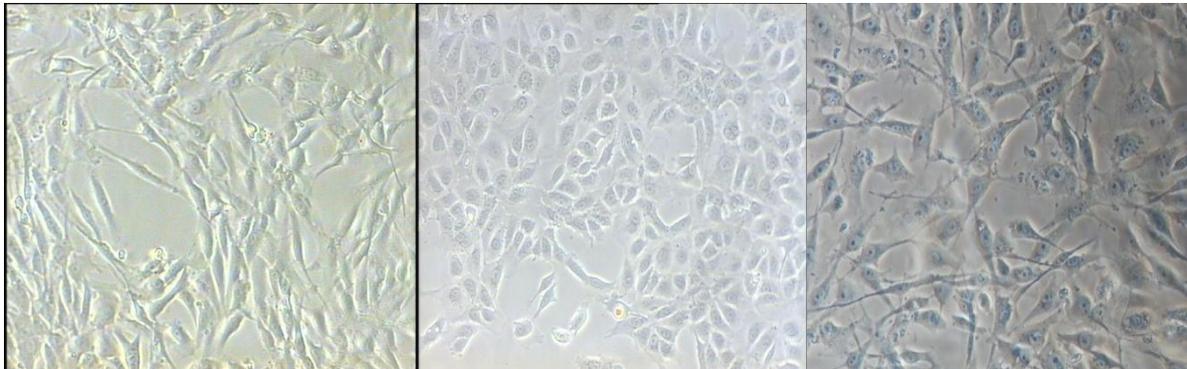

Figure 2.
Phase-contrast micrographs showing STAV-FCS cells (left panel) with elongated fibroblast-like phenotype, STAV-AB cells (middle) with polygonal epitheiloid phenotype and B6FS fibrosarcoma cells (right panel) with fibroblast-like appearance.

Plasmids

The vector used in these experiments, pEGFP-N1, was obtained from BD Bioscience (Clonthech, Palo Alto, CA, USA). It contains a gene encoding for the enhanced green fluorescence protein (EGFP) and a geneticin resistance gene. Geneticin was used as selective antibiotic for eliminating the non-transfected cells due to their lack of expression of the geneticin resistance gene (fig. 3).

Syndecan-1 constructs (FL-sdc1-EGFP, 78-EGFP, 77-EGFP, and RMKKK-EGFP) used for transfection, were obtained from Laszlo Szilák Labour Ltd (Szeged, Hungary) (fig. 4). In addition, two syndecan-1 transfectants lacking the EGFP were used in order to avoid interference with the sub-cellular localization of syndecan-1 (fig. 5). Stable transfectants were cultured with 600 units of geneticin, 200 units of Fungizone, 250 UG/ml Amphotericin B [*GIBCO Introgen*] and 10.000 µg/ml Penicillin/Streptomycin [*GIBCO Introgen*].
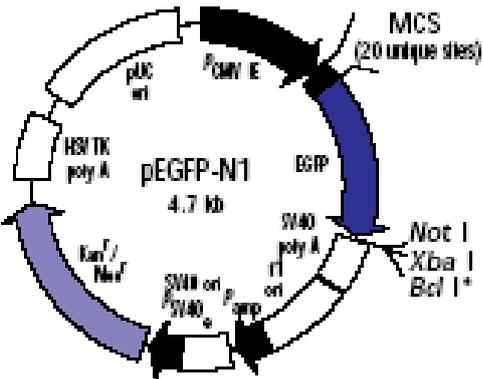

Figure 3.
Vector used for syndecan-1 transfection. The blue-purple colour reflects the geneticin gene and the dark blue colour represents the EGFP gene.
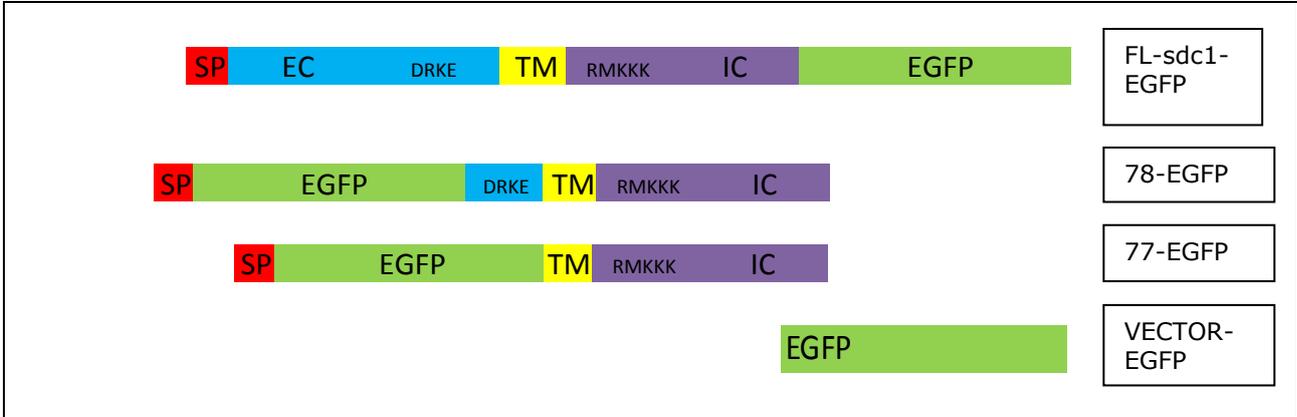

Figure 4.
SP= Signal peptide EC= Extracellular domain TM= Transmembrane domain
IC= Intracellular domain

FL-sdc1-EGFP:corresponds to full-length-syndecan-1 with the EGFP sequence at the cytoplasmic terminal (C-terminal).78-EGFP:Truncated construct lacking the EC with exception of the oligomerization sequence (DRKE). The EGFP sequence is next to the TM. 77-EGFP:construct lacking the EC. RMKKK-EGFP:This is the smallest construct and the short stretch of amino acids encodes for the nuclear localization signal. VECTOR-EGFP:An empty vector containing no syndecan-1, only EGFP. This construct was used as the control.
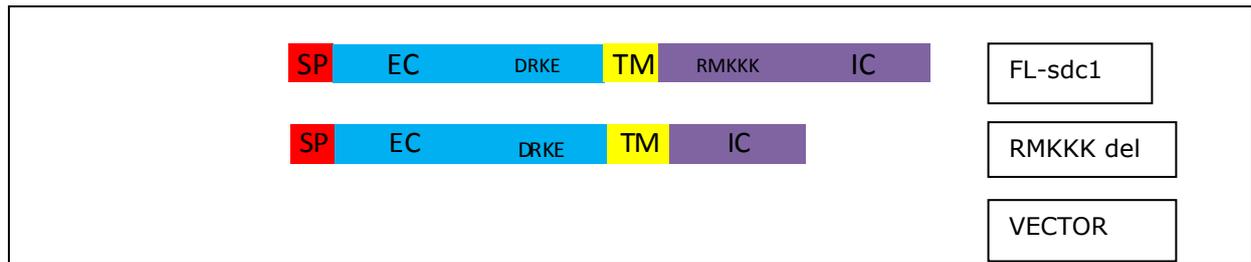
SP EC DRKE TM RMKKK IC   FL-sdc1

SP EC DRKE TM IC   RMKKK del

                                     VECTOR

Figure 5.

FL-sdc1:corresponds to full-length-syndecan-1 construct lacking the EGFP gene. RMKKK del: The RMKKK deletion mutant is FL-sdc1 lacking the RMKKK nuclear localisation signal VECTOR: Empty vector control.

Transfection

Cells were transfected with the above syndecan-1 constructs using the Effectene Transfection Reagent [*Qiagen GmbH, Hilden, Germany*], according to the manufacturer's instructions. The effect of transfection was followed by fluorescence microscopy after 18, 24 and 48 hours. After 48 hours the selection for transfected cells started by adding 200 units of geneticin [*Invitrogen, Karlsruhe, Germany*]. After approximately one week the dose was increased to 400 units.

Real-time Quantitative Polymerase Chain Reaction (Rt-Q-PCR)

In order to quantify the syndecan expression on RNA level, Rt-Q-PCR was conducted. The PCR method then allowed us to rapidly amplify the cDNA and by detection of fluorescence from the fluorophore SYBR$^{®}$ Green in real time, the quantity of the DNA could be achieved. A standard curve was used and the ratio between the arbitrary values of the different transfected cells and the reference gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH), gave us information of how the transfection has affected the syndecan expression in the different samples.

RNA isolation

RNA isolation was performed by using the High Pure RNA Isolation Kit [*Roche, Mannheim, Germany*] according to the manufacturer's instruction. Briefly, cells were trypsinized, washed in PBS and centrifuged at 400 g for 5 minutes. The pellet was first resuspended in PBS and mixed with a lysis/binding buffer. The sample was then centrifuged at 8000 g for 15 seconds followed by resuspension in a DNase incubation buffer. DNase 1 mix was added to degrade the DNA and the sample was incubated for 15 minutes at room temperature. The cells were washed with buffer 1 and centrifuged as above. They were washed again with buffer 2 and centrifuged at 13000 g for 2 minutes. The elution buffer was added to the samples to elute the RNA by centrifugation at 8000 g for 1 minute. The RNA was diluted and transferred to a glass photometric plate and the concentration and purity could be measured in an ELISA reader at 260-280 nm. In the B6FS cell line triplicates from different isolates were used.

STAV-AB cell line was used as a pilot experiment and only one isolate was used.

cDNA synthesis

For cDNA synthesis the Omniscript Reverse Transcription Kit [*Qiagen, Hilden, Germany*] was used. A master mix was prepared according to the Omniscript Reverse Transcription handbook, 2µg RNA was added and the samples were well mixed and incubated for 60 minutes at 37 °C followed by Rt-Q-PCR.

Rt-Q-PCR

A master mix containing RNase free water, Platinum ® SYBR ® Green qPCR SuperMix-UDG [*Invitrogen, Karlsruhe, Germany*], forward and reverse primers of the syndecan used (1, 2, 3, or 4) together with FITCH Dye (ratio 1:1000 with RNase free water) was prepared. For reference, GAPDH primers were used. The master mix was then mixed, gently but well, and aliquoted to the cDNA. The mix was applied in a 96-well-PCR plate in triplicates and put in the PCR machine that was programmed for Standard Cycling Program for ABI Instruments (40 cycles of 95 °C, 15 seconds and 60°C, 30 seconds). The expression of syndecan on RNA level was calculated as fold change based on the cycle threshold (Ct) values from the Rt-Q-PCR.

Immunocytochemical analysis and confocal laser microscopy

The cells surviving the geneticin selection were expected to overexpress syndecan-1. To study syndecan-1 expression on protein level the cells were stained with antibodies against syndecan-1 followed by confocal laser microscopy. The confocal microscope allows a higher resolution than a phase contrast/fluorescence microscope. The source of light is a focused argon-krypton laser that passes through a pinhole and scans the focal plan in virtual sections. A computer registers exclusively the fluorescence signal coming from the focal plan. The method is used to study subcellular detection and distribution of different proteins [3D].

When the stably transfected cells had reached about 60 % confluency, they were first trypsinized to detach and then seeded on glass slides. These cultures were then incubated for 48 hours followed by immunochemical staining. Cells were fixed with prewarmed
3 % paraformaldehyd [*Kebolab, Spånga, Sweden*] for 10 minutes at 37 °C followed by permeabilisation with 0.1 % Triton x-100 [*Sigma-Aldrich Chemie, Steinheim, Germany*] for 10 minutes to allow antibody reagents to enter the cell.

The unspecific binding sites were blocked by applying 3 % Goat serum [*Dako Cytomation, Glostrup, Denmark*] for 30 minutes at room temperature. The cells were stained with the CD138 mouse anti-human syndecan-1 primary antibody [*Serotec, Oxford, UK*] in 1 % goat serum, or for the

negative control with mouse IgG1 [*Dako Cytomation, Glostrup, Denmark*]. The reaction was performed for 1 hour at room temperature in the dark.

The secondary antibody Alexa 568 goat anti-mouse IgG1 (γ-1) [*Molecular probes, Oregon, USA*] was allowed to react for 30 minutes at room temperature in the dark. Nuclei were stained with Hoescht 33342.The slides were mounted with cover glass using Dako Mounting medium [*Dako cytomation, California, USA*] allowing polymerization at 4°C for 30 minutes.

Cell proliferation

To study the proliferation rate of the syndecan-1 overexpressing cells, a colorimetric assay using the WST-1 cell proliferation reagent [*Roche, Mannheim, Germany*] was preformed. This is a sensitive and accurate method well suited for measuring the amount of metabolically active cells.

Cells change their behaviour depending on the growth density. An overcrowded area results in inhibition of cell proliferation. Standardized constant growth proportions can be obtained by optimization, which is recommended prior to cell proliferation assay.

Optimization of number of cells
Non-transfected cells were used for optimization. The cells were trypsinized to detach, dilution series were prepared and counted in an automatic cell counter to correspond to 30 000, 10 000, 3000, 1000 and 300 cells, respectively. The cells were then seeded in a 96-well-plate and incubated for 4 respectively 24 hours including a 4 hour incubation time of WST-1. The absorbance 450- 650 nm was then measured in an ELISA microplate reader to quantify the cell number.

WST-1 cell proliferation
The optimal number of cells was seeded in a 96-well-plate to give maximal proliferation according to the optimization experiment. Transfected STAV-AB and B6FS cells were incubated for 4, 24, 48 and 72 hours including a 4 hour incubation time of WST-1 prior to absorbance measurements.

Cell cycle analysis with fluorescence activated cell sorting (FACS)

The cells were seeded 24 hours prior to treatment and cultured as described in the "Cell culture" section. They were trypsinized and centrifuged at 400 g for 5 minutes. $0.5 – 2 \times 10^6$ cells were suspended in 5 ml 0.01 M PBS and again centrifuged as above. Cells were than fixated in 70% EtOH. To avoid aggregation of the cells they were kept on ice and vortexed frequently. After fixation over night in 4°C the cells were washed in PBS and centrifuged at 400 g for 5 minutes followed by DNA staining in 500 µl staining solution consisting of 50 µg/ml propidium iodide [*Sigma-Aldrich®*] and 100 µg/ml RNase A [*Sigma-Aldrich®*]). The cells were transferred to FACS tubes and incubated for 30 minutes in 37°C followed by FACS analysis.

Quantification of total heparan sulfate content by FACS

Flow cytometry allowed us to separate cell populations with different properties. By this method fluorescence of the cells was detected when cells in suspension passed through a laser beam. This way cells with fluorescence got separated from cells without fluorescence. By using FACS analysis we could separate the transfected STAV-AB cells from the non-transfected cells (gating) by measuring the green fluorescence that the transfected cells expressed. From this we could study how the heparan sulfate (HS) expression had been affected by the transfection. The experiment was performed with and without Saponin permeabilisation. Saponin temporarily opens up the cell membrane without harming the cells and allows detection of HS at intracellular locations.

Transfected and non-transfected cells from the STAV-AB cell line were first washed with PBS. To detach the cells from the 75 cm³ culture flask the cells were incubated with 5mM EDTA [*Merck, Darmstadt, Germany*] in PBS for 15 minutes at 37 °C. The cell structures were kept by fixating them in 1% buffered Formalin [*Apoteksbolagen, Gothenburg, Sweden*] for 15 minutes at room temperature.

The cells were permeabilised for 15 minutes with 0.1% Saponin [*Fluka, Buchs, Switzerland*] in PBS at room temperature so that the applied antibody could enter the cell, and 0.6% BSA was added to prevent cell clustering.
Equal amounts of a small volume from each tube were distributed to three new tubes. To the first tube we added nothing and it contained only the cells. To the second tube we added the negative control FITCH conjugated mouse IgM [*Bioscience, Cambridge, UK*]. To the third tube we added the primary FITCH conjugated HS antibody [*Seikagaku, Tokyo, Japan*]. The cells were incubated for 15 minutes at room temperature. Then the cells were washed with 0.1% Saponin in PBS and 0.6% BSA followed by centrifugation at 400 g for 5 minutes. The cells were resuspended in 0.1 % Saponin in PBS and 0.6 % BSA and tubes two and three were incubated with the secondary antibody donkey anti-goat IgG Alexa 568 [*Molecular Probes, Oregon, USA*] (1:200) for 15 minutes in the dark at room temperature. The last washing step in 0.1 % Saponin in PBS and 0.6 % BSA followed by centrifugation at 400 g for 5 min. The cells were resuspended in PBS and now the FACS analysis could be performed.

RESULTS

Transfection

The stable transfection was successful with three of the constructs after selection with geneticin.

After 18 and 24 hours post transfection fluorescence was detected in most of the cells, but strongest in the cells transfected with the RMKKK-EGFP and FL-sdc1-EGFP plasmids for both STAV-AB and STAV-FCS cells (fig.6). After 48 hours the selection with geneticin started and a decrease was observed in the fluorescence in all of the samples. The STAV-FCS cell sub-line did not show any sign of fluorescence or proliferation after this time-point and was not involved in further experiments.
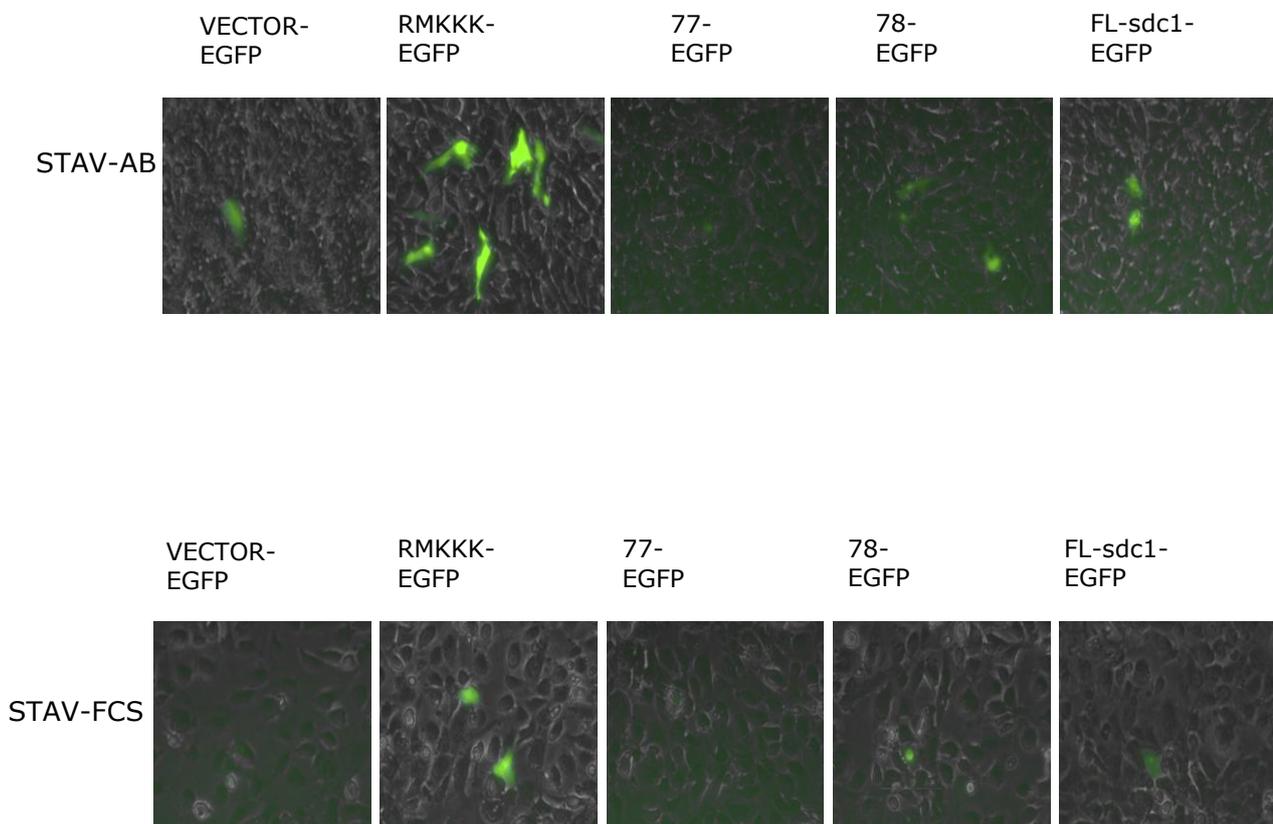

Figure 6.
Transfected cells documented after 18 hours by time-laps fluorescence microscopy, when the fluorescence was clearly visible.

Weeks after transfection the number of cells had decreased and little fluorescence was seen in the remaining STAV-AB cells. Although colonies were observed in the surviving cells transfected with the RMKKK-EGFP, 77-EGFP and VECTOR-EGFP constructs. Finally, 3 months after transfection the same observation was made.

Detection of overexpression of syndecan-1 at RNA level

To assess the level of overexpression of syndecan-1 at RNA level RNA isolation, cDNA synthesis and Rt-Q-PCR was conducted. The RNA for each transfectant was pure enough to proceed with cDNA synthesis since they all were in the range of 1.75-2.17.

Rt-Q-PCR showed an overexpression of syndecan-1 in the transfectants in both cell lines. There was a 7-fold increase of syndecan-1 in the cells transfected with the 77-EGFP construct and there was a 5-fold increase for the RMKKK-EGFP compared to the control VECTOR-EGFP (fig. 7).
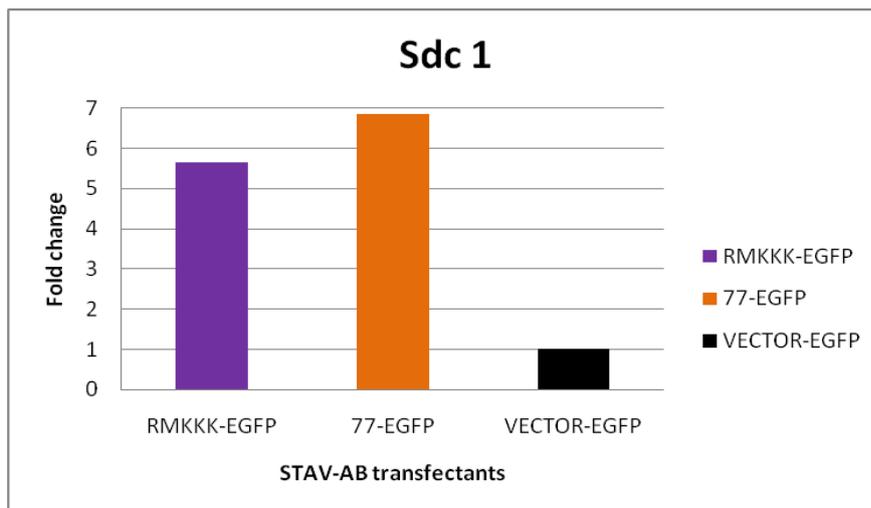

Figure 7.
Expression of syndecan-1 displayed as the fold change for the different transfectants compared to their control VECTOR-EGFP.

Syndecan-1 was approximately 4-fold increased in FL-sdc1 transfeced cells, and 2-fold increase in RMKKK deleted construct compared to the control VECTOR (fig. 8).
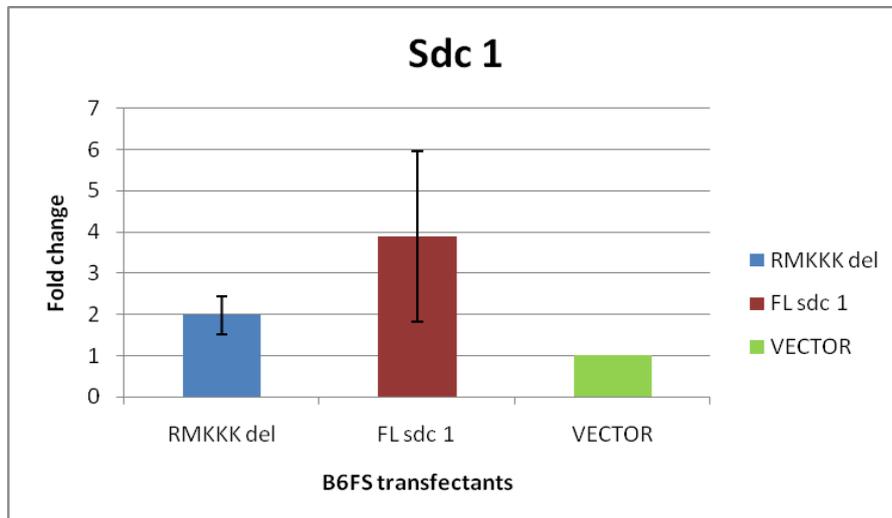

Figure 8.
Expression of syndecan-1 displayed by the average of three experiments for the different transfectants and control. The bars indicate the standard deviation.

Evaluation of overexpression of syndecan-1 at protein level

Syndecan-1 was observed in the cytoplasm showing an even distribution for the transfectants by confocal laser microscopy after immunocytochemical staining (fig.9). In the 77-EGFP cells the signal was more intense comparing to the others. The cells transfected with the 77-EGFP plasmid had changed their morphology to a more elongated fibroblastic phenotype. Only faint green fluorescence of the cells was detected.

VECTOR-EGFP   77-VECTOR   RMKKK-VECTOR

Figure 9.
Images from immunocytochemical staining. There were signals of syndecan-1 expression in the transfected cells. The red signal indicates all cells expressing syndecan-1.

Effects of overexpression of syndecan-1 on cell proliferation and cell cycle distribution

<u>Optimization</u>

Optimal growth condition and maximal proliferation rate was seen in wells that contained 3000 and 10 000 cells in the optimization experiment (fig. 10). The number of cells optimal to use in a cell proliferation assay is therefore around ~5000 cells. Higher cell number resulted in saturated values and growth inhibition.
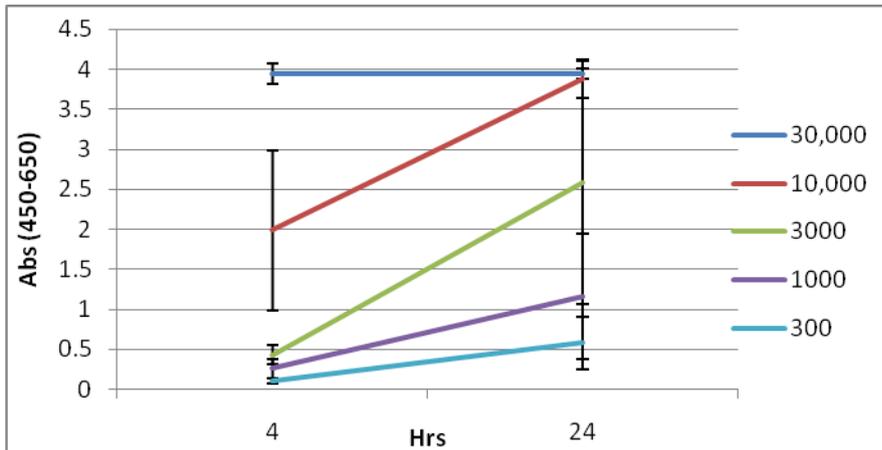

Figure 10.
Optimization of the number of cells. Between 5000 and 10 000 cells is preferably to use.

The truncated 77-EGFP and RMKKK-EGFP syndecan-1 constructs, showed decreased cell proliferation when compared to the corresponding VECTOR-EGFP control. The effect was most evident after 24 hours. The most pronounced effect was seen in RMKKK-EGFP corresponding to the nuclear localization signal (fig. 11).
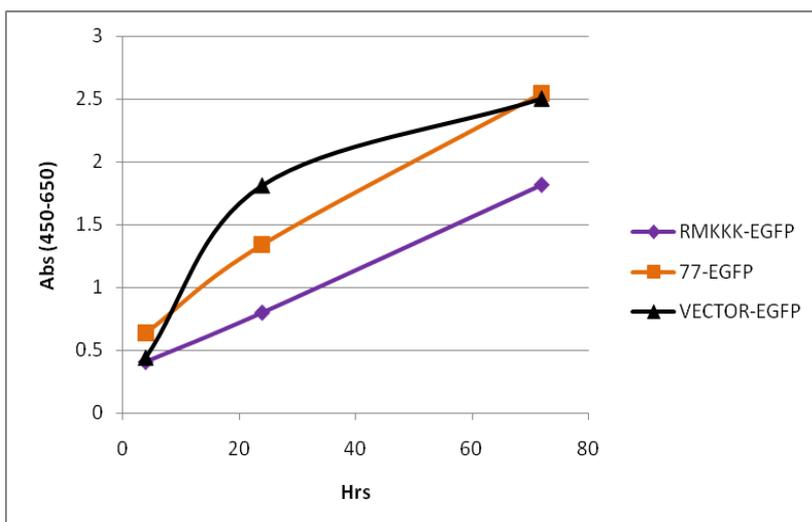

Figure 11.
The average of three independent cell proliferation experiments in STAV- AB cells transfected with the truncated 77-EGFP and RMKKK-EGFP syndecan-1 constructs and the VECTOR-EGFP control.

The B6FS tranfectants show the same tendency as the STAV-AB transfectants were the FL-sdc1 and RMKKK del showed a lower rate of proliferation when compared to the control VECTOR (fig. 12).
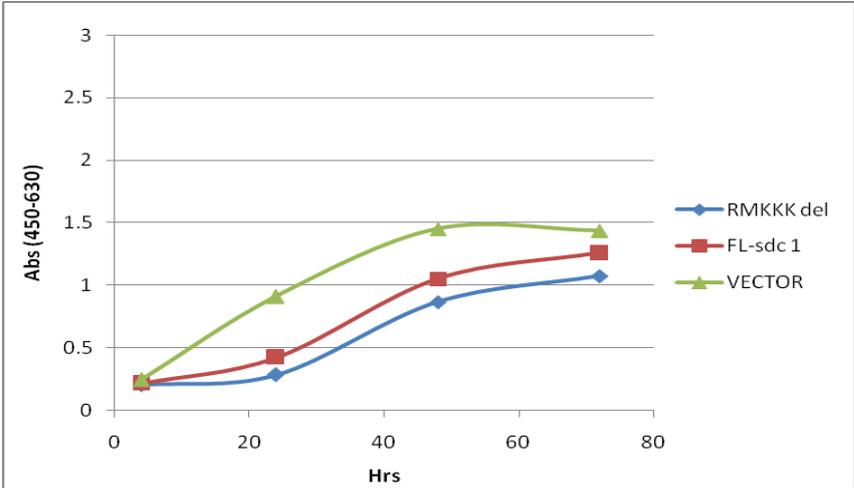

Figure 12.
The average of three independent experiments showing the proliferation of B6FS cells transfected with the FL-sdc1 and RMKKK del construct and their corresponding control.

The results from the cell proliferation assays are reflected also in the cell cycle analysis pointing to a tendency of accumulation of cells in the G0/G1 phase of the cell cycle. The RMKKK del shows a slightly higher accumulation compared to the control, but the results are not statistically significant due to high variability between the experiments (fig. 13).
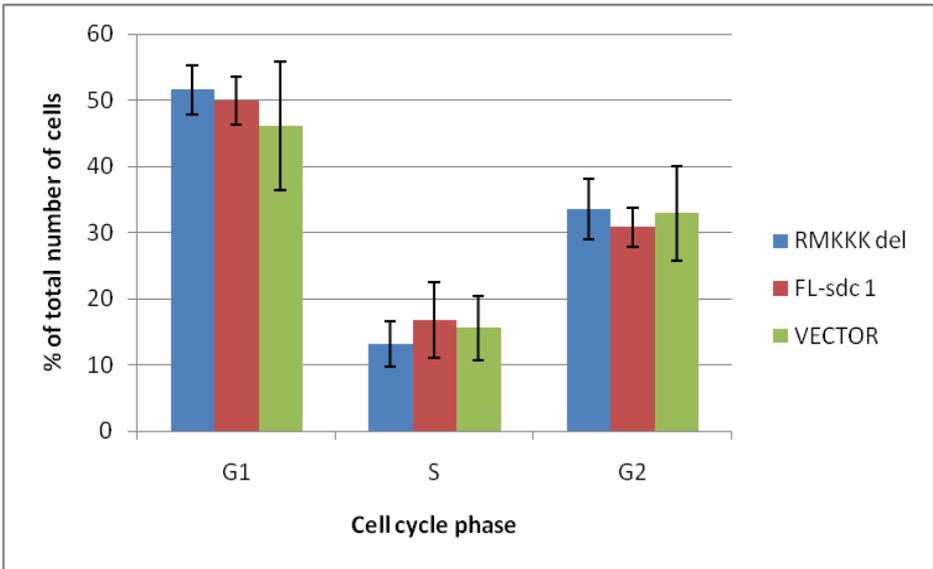

Figure 13.
Cell cycle analysis at 24 hours.

Effects of overexpression of syndecan-1 on the other syndecan family members

Overexpression of syndecan-1 in the STAV-AB cell line resulted in an upregulation of syndecan-1 and syndecan-4. There was only a minor increase of syndecan-2 as compared to the control (fig. 14).
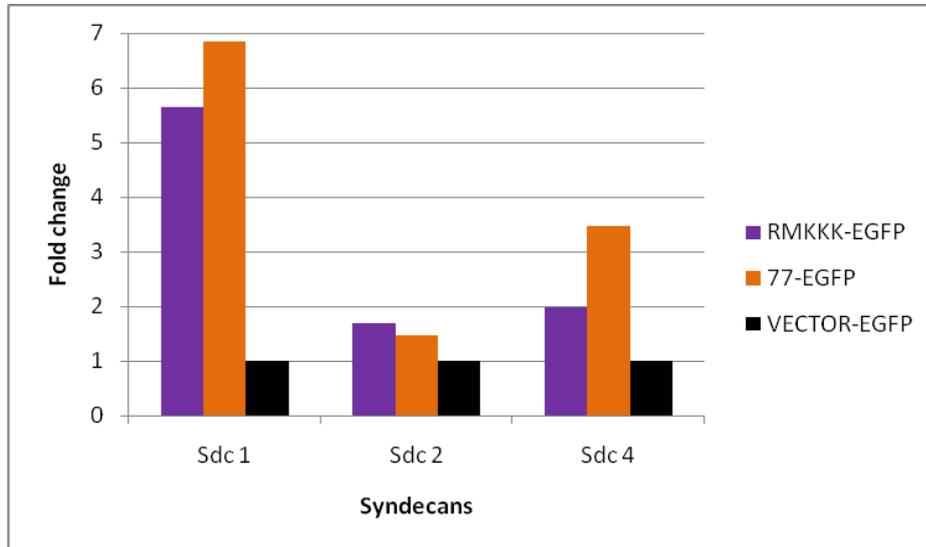

Figure 14.
Rt-Q-PCR on the STAV-AB cell line. Syndecan-2 did not seem to be significantly affected by the syndecan-1 overexpression, but syndecan-4 was upregulated compared to its control.

In the B6FS cell line syndecan-2 and -4 were both downregulated by the overexpression of syndecan-1. Syndecan-2 expression decreased in both full-length syndecan-1 and RMKKK deletion mutant transfectants. Syndecan-4 was also downregulated particularly in the RMKKK deletion transfectant comparing to the control VECTOR (fig. 15).
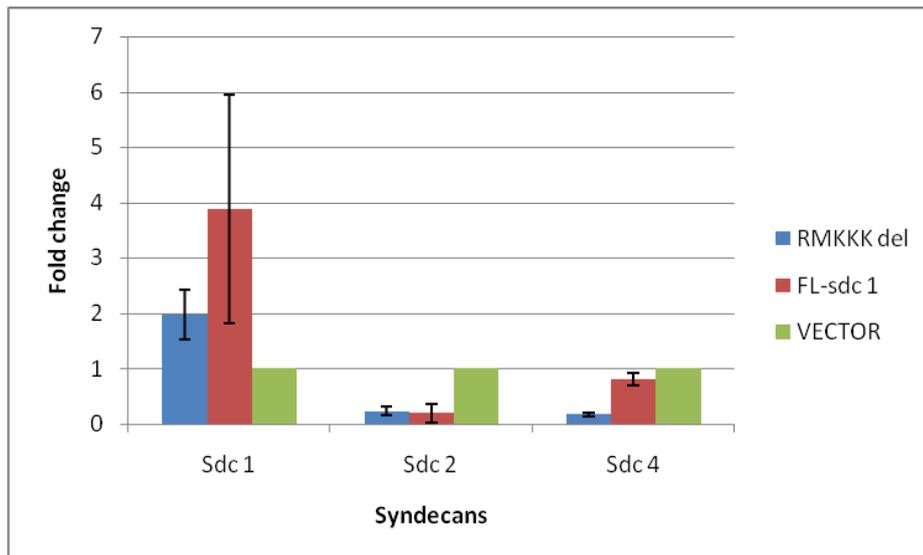
Figure 15.
Results from Rt-Q-PCR on the B6FS cell line. Syndecan-2 and 4 are downregulated due to the syndecan-1 overexpression comparing to their controls.

Cell surface and total syndecan expression by heparan-sulfate content chains are present in all members of the syndecan family. To assess how syndecan-1 overexpression influenced the overall syndecan level, we measured the HS content first on the cell surface, then the intracellular syndecans, by opening the pores of cell membrane with saponin treatment. In the experiment with cells not treated with saponin, all transfectants and controls express the similar levels of HS (fig 16).

In the experiment where the cells were treated with saponin, we could see an overall upregulation of HS in both the transfectants RMKKK-EGFP and 77-EGFP comparing to the control. The upregulation was most prominent in the RMKKK-EGFP construct comparing to 77-EGFP (fig. 17).
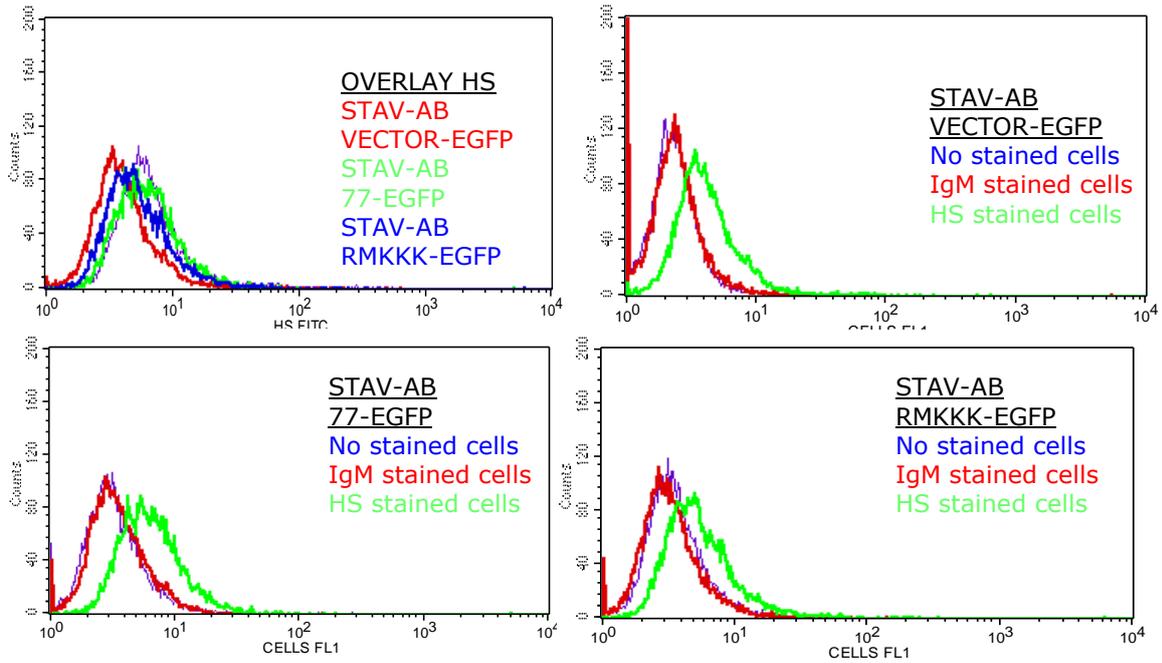
Figure 16.
Cells not treated with saponin and analysed by FACS. The graphs show that there is no difference in HS expression between the transfected STAV-AB cells and the control.
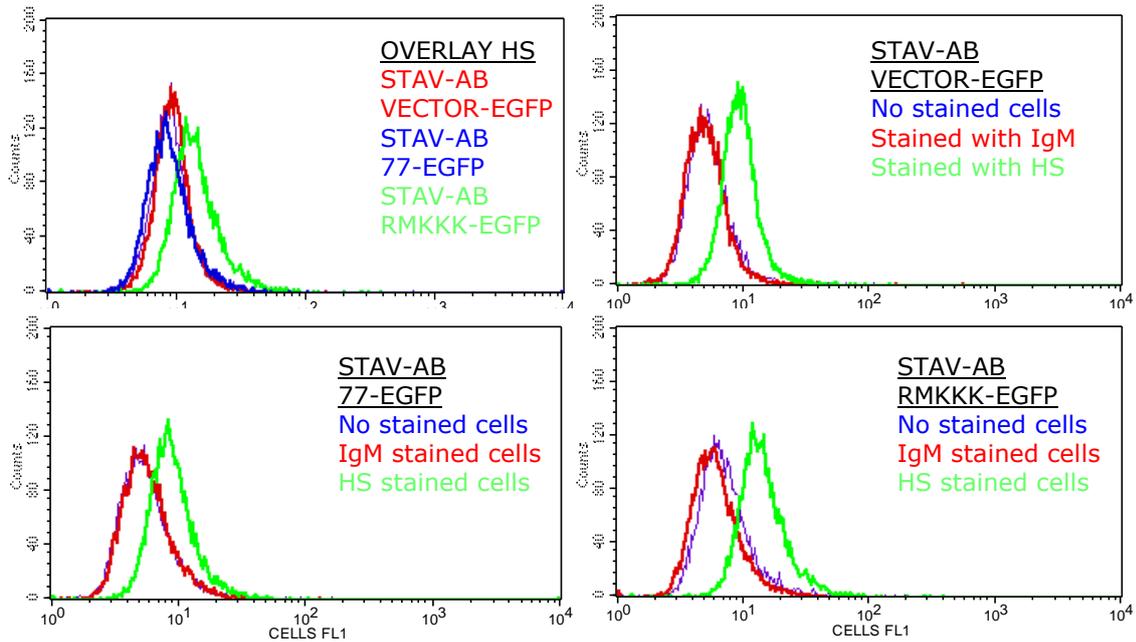

Figure 17.
Saponin treated cells and analysed by FACS. The graphs show that there is difference in HS expression between the transfected STAV-AB cells and comparing to the control.

DISCUSSION

Tumor development and tumor progression can vary extensively regarding the deviation from normal growth to abnormal growth. Highly aggressive mesenchymal tumors such as malignant mesotheliomas and fibrosarcomas primarily invade the underlying tissue and later they migrate from the primary tumor by entering blood-and lymph vessels and establish new colonies at distant locations[19]. One important part of tumor progression relates to the autonomous replication of cells. Studies of tumor cell proliferation are therefore essential for the understanding of the malignant properties of the respective tumors.

Syndecan-1 is overexpressed in many epithelial tumors and downregulated in dedifferentiated cancers or sarcomatoid tumor components. The level of syndecan-1 expression correlates to their proliferation and differentiation[18]. In malignant mesothelioma and fibrosarcoma the syndecan-1 expression is generally low and upregulation of syndecan-1 may influence their proliferation[11]. The focus of this study is therefore to understand the role of syndecan-1 in the proliferation of malignant mesenchymal tumors by overexpressing syndecan-1 and assessing the proliferation of two mesenchymal cell lines with low endogenous syndecan-1 expression level on their cell surface.

In our experiments full-length syndecan-1 transfectants displayed a 4-fold overexpression of syndecan-1 which resulted in a decrease in cell proliferation. Similar decrease was also observed in benign fibroblasts[18] and in mouse mammary epithelial cells[19] proliferation, following syndecan-1 expression. In contrast, overexpression of syndecan-1 increased cell proliferation in human endometrial cancer[20], hepatocytes[21] and the HT1080 fibrosarcoma cell line[22], indicating a cell type specific effect which probably also depends on the basal level of syndecan-1 and the proteoglycan profile of the cells.

Most of the studies adressing the function of syndecan-1 are focusing on the ectodomain and there are just a few publications dealing with the role of the transmembrane or cytoplasmic domains of syndecan-1 in tumor cell proliferation. In my study, I also investigated the effects of overexpression of truncated syndecan-1 proteins on cell proliferation. The construct lacking the ectodomain had a lower proliferation rate comparing to the control. Similar result was found in a publication were the proliferation of mesenchymal cells was tested[13]. Interestingly, in hepatocytes proliferation is induced due to the lack of the C-terminal of the syndecan cytoplasmic tail on a truncated version[23], highlighting in concordance with our results the importance of the intracellular portion of syndecan-1 in modulating cell growth.

The deletion of RMKKK nuclear localization signal in the syndecan-1 protein caused an even more pronounced inhibition of proliferation, although the overexpression of this construct was lower compared to the full-length syndecan-1.

Syndecan-1 was first considered to be a cell surface molecule. However, recent studies have shown that the syndecan molecule get translocated to the nucleus on behalf of the RMKKK nuclear localization signal, and it is therefore vital for the syndecan molecule[11, 12, 13]. This event has also proven to decrease proliferation of the mesenchymal tumors studied in this project. Other studies also support the functional role of syndecans in the nucleus via inhibition of topoisomerase I and histone acetyltransferase[23, 24].

A cell cycle analysis after 24 hours showed a small accumulation of the transfected cells in the G0/G1 phase of the cell cycle but these differences in cell cycle analysis were not significant. In a similar study conducted on mesenchymal cells, it was shown that cells overexpressing syndecan-1 have a prolonged S-phase which was in correlation with the slower growth of these cells[13].

All cells have at least one member of the syndecan family and most cells express multiple forms. There is a distinct pattern of syndecan expression that characterizes individual cell types and tissues. Several studies are showing that proteoglycans are important in differentiation, infiltration and angiogenesis of various malignant tumors[26, 27]. The syndecan expression profile can influence the proliferation as well. Therefore we studied the effect of overexpression of syndecan-1 on the other syndecans and we found that it has a pronounced effect of the other syndecan family members where syndecan-2 and -4 were downregulated. Especially syndecan-2 was affected by both the full-length syndecan-1 and the RMKKK deleted construct. Syndecan-4 showed similar result for the RMKKK deletion mutant. In contrast, the 77-EGFP and RMKKK-EGFP truncated constructs resulted in an elevated expression of syndecan-4 and only slightly higher values in syndecan-2 expression comparing to their controls. Taken together our data show that the effect of overexpression of syndecan-1 is cell type specific and need to be further investigated.

Based on my observation that syndecan-1 overexpression influences the other members of the syndecan family, cells were stained in parallel to detect their total heparan sulfate content as a measure of their proteoglycan expression. The expression of syndecan-bound heparan sulfate was studied by FACS and indicated an upregulation for heparan sulfate when measuring internal- and membrane bound syndecans simultaneously.

Recent studies have shown that the RMKKK sequence is the nuclear localization signal and that the syndecans get translocated into the nucleus and can accumulate in the nucleus depending on the presence of tubulin[11, 12, 13]. This event has also proven to hamper the proliferation of the mesenchymal tumors studied in this project [12, 13].

Syndecan-1 may also serve as a prognostic marker in various cancer-types, and it may also provide information on tumor progression.

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