The complex role of syndecan-4 in skeletal muscle: myogenesis and oncogenesis

Ph.D. Thesis

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LIST OF PUBLICATIONS

1. List of full papers directly related to the subject of the thesis:

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II. Keller-Pinter A, **Szabo K**, Kocsis T, Deak F, Ocsovszki I, Zvara A, Puskas L, Szilak L, Dux L. Syndecan-4 influences mammalian myoblast proliferation by modulating myostatin signaling and G1/S transition. *FEBS Lett. 2018 Sep;592(18):3139-3151.* **[IF: 2.675] D1**

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II. Becsky D*, **Szabo K***, Gyulai-Nagy S, Gajdos T, Bartos Z, Balind A, Dux L, Horvath P, Erdelyi M, Homolya L, Keller-Pinter A. Syndecan-4 Modulates Cell Polarity and Migration by Influencing Centrosome Positioning and Intracellular Calcium Distribution. *Front Cell Dev Biol. 2020 Oct 15;8:575227.* [IF: 5.87] Q1
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1. INTRODUCTION

1.1. Muscle development and regeneration

The process of muscle fibre development is called myogenesis. During embryonic development it is controlled by precise molecular signals, transcription and growth factors that result in the formation of a heterogeneous musculature. Myogenesis is initiated by stem cells in the spinal cord of the embryo. Stem cells can be transformed into cells characteristic of any tissue in response to the appropriate chemical signal. Mononuclear myoblasts accumulate around the site of striated muscle formation. Some of these form the embryonic muscle, while a distinct cell lineage gives rise to satellite cells from which the regeneration of injured muscle can be initiated. By understanding the process of skeletal muscle regeneration we might have the possibility to improve it following sport injuries, muscle diseases or during aging.

1.2 Molecular mechanism of skeletal muscle tissue formation

The transcription factors that regulate myogenesis and muscle differentiation include the members of the MyoD (myoblast determination protein 1) family [MyoD, Myf5, MRF4, and MyoG (myogenin)], also known as myogenic regulatory factors (MRFs). MRFs appear in distinctive spatial and temporal patterns during embryonic development and regeneration of striated muscle.

Of the many factors, myostatin (GDF8, growth differentiation factor 8) plays an important role in regulating muscle development and regeneration by inhibiting myoblast proliferation and differentiation. It also prevents hypertrophy and hyperplasia of skeletal muscle tissue. It is associated with two types of activin receptors, ActI and ActIIa/b. These receptors form heteromers and are phosphorylated upon binding of myostatin as a ligand, a process that leads to phosphorylation of Smad2 and Smad3 molecules, as miostatin acts through the Smad-mediated signal transduction pathway. In this way, it inhibits the action of MyoD and MyoG thus inhibiting muscle cell commitment and differentiation. However, in addition to the Smad pathway, it can also exert inhibitory effects on certain members of the insulin-like growth factor 1 (IGF1) signaling pathway. In addition, it activates p21 protein leading to inhibition of Cdk2 (cyclin-dependent kinase) and, consequently the unphosphorylated Rb inhibits the transition of G1/S phases of the cell cycle. Therefore, muscle regeneration and differentiation do not occur.

1.3. Syndecan family

Syndecans (SDCs) are type I transmembrane proteoglycans that, in addition to their structural function, also play a significant role in signal transduction. Four members of the family in vertebrates are known. In terms of their structure, they consist of an N-terminal variable extracellular domain (ectodomain), a highly conserved transmembrane, and a C-terminal intracellular domain. Glycosaminoglycan (GAG) side chains composed of repeating disaccharide molecules are attached to the N-terminal domain. These GAG chains are heparan sulfates for SDC2 and SDC4, but chondroitin sulfate side chains are also found in SDC1 and SDC3 beyond the heparane sulfates. SDC1 is mainly expressed in epithelial and plasma cells, SDC2 in mesenchymal tissues, fibroblasts, SDC3 in neuronal tissue, skeletal muscle, while SDC4, unlike other members of the family, is universally expressed in virtually all nucleated cell types.

1.4. SDC4

Due to the transmembrane structure, SDC4 plays an important role in bidirectional communication between the cell and the surrounding matrix. Among other things, it plays a role in the formation of focal adhesions, cell migration, wound healing, and the process of angiogenesis, inflammation, and tumor spread. SDC4 can also function as receptors and co-receptors. SDC4 binds growth factors, chemokines, enzymes through heparan sulfate chains, and is involved in the formation of cell-cell adhesions, or even indirectly binds to actin filaments and is involved in altering the cytoskeletal skeleton.

The heparan sulfate chains of the extracellular domain are able to bind FGF2, thus functioning as a co-receptor for the FGF receptor. The intracellular domain regulates Rac1 and binds to the actin cytoskeleton via α-actinin. It influences cytokinesis, is involved in vesicular transport processes, and affects intracellular calcium levels through TRPC channels.

SDC4 is a marker of activated and resting satellite cells, and developing skeletal muscle shows significant SDC4 expression. Furthermore, during soleus muscle regeneration, SDC4 mRNA expression was increased.

In many tumor types, SDC4 expression is unregulated, and in most cases SDC4 is upregulated. Changes in SDC4 expression levels can be observed in several tumor types, and it serves as a prognostic marker, such as in breast cancer, glioma, melanoma, liver cancer, and osteosarcoma.

Mice deficient in SDC4 transmembrane proteoglycan are less responsive to postnatal and injury stress situations and have prolonged wound healing. It is known that Rac1 levels in

fibroblasts of SDC4 knockout (KO) mice are elevated, angiogenesis is impaired, satellite cell activation and proliferation, MyoD expression, and muscle regeneration.

SDC4 influences Rac1 activation and accumulates active Rac1 at the leading edges of migrating cells, thus allowing the formation of membrane spurs that are essential for the fusion process.

1.5. Myoblast fusion and actin cytoskeleton rearrangement

Cell fusion is a phenomenon that occurs in many processes, not only during myogenesis, but also during the formation of osteoclasts, syncytiotrophoblasts, and tumour cells. For fusion to occur, the morphology of the cells must first change, the fibroblast-like, star-shaped appearance must change to a spindle-like, elongated shape. During fusion, the plasmamembrane forms protrusions called lamellipodia or filopodia.

During fusion, a continuous rearrangement of the actin cytoskeleton is observed, with several molecules involved in the regulation of this rearrangement. The key intracellular components that act downstream of cell adhesion molecules to regulate the continuous and dynamic rearrangement of the actin cytoskeleton are members of the Rho family of small GTPases, the best characterized members of which are RhoA, Rac1 (Ras-related C3 botulinum toxin substrate 1), and Cdc42. Small GTPases act as molecular switches. Several studies suggest that the Rac1 small GTPase is a central regulator of myoblast fusion in Drosophila and it has also been reported that Rac1 and Cdc42 are essential for myoblast fusion in vertebrates. Rac1-GTP levels are increased at the site of fusion and constitutively active Rac1 induces myoblast fusion [68]. Conversely, because active RhoA antagonizes Rac1-GTP, expression of constitutively active RhoA decreases myoblast fusion.

The actin cytoskeleton is a dynamic system, it is constantly remodelled during polymerisation and depolymerisation. The Arp2/3 complex and Dia, a formin, are responsible for the nucleation of actin polymerization. In a Rac1 and Cdc42-dependent manner, the Arp2/3 complex initiates new filament formation by attaching to the side of the pre-existing actin filament in a Rac1- and Cdc42-dependent manner, by forming a 70° angle with the original filament, while the Rho effector Dia elongates actin filaments linearly.

1.6. Rhabdomyosarcoma

Rhabdomyosarcoma is the most common soft tissue sarcoma of skeletal muscle origin in children. Its incidence in young adults < 20 years of age in the USA is 4.4/1 million per year. Its traditional classification system is based on histological observations, which have been used

to identify four groups: embryonal rhabdomyosarcoma and its botryoid variant, pleomorphic and alveolar rhabdomyosarcomas. Then, in 2013, in the light of molecular biology results, a new classification system was published, which separates only two main groups: fusion positive and fusion negative rhabdomyosarcomas. A tumour is fusion positive if either the PAX3-FOXO1 fusion protein resulting from the t(2;13)(q35;q14) chromosomal translocation or the PAX7-FOXO1 protein resulting from the t(1;13)(p36;q14) translocation is detected in the cells. In all other cases, fusion is considered negative in rhabdomyosarcoma.

Although the survival of patients with rhabdomyosarcoma has improved over the past 40 years, the prognosis of metastatic or recurrent cases remains unfavourable. Therefore, additional research is needed to further elucidate the molecular background of the disease.

2. AIMS OF THE THESIS

It is known from the literature that SDC4 gene knockout mice have impaired skeletal muscle regeneration and elevated Rac1 GTPase activity. Rac1 is also known to play an important role in the fusion of mammalian myoblasts, including the rearrangement of the actin cytoskeleton via PAK1, which is a key determinant of cellular resilience. There is also evidence that high SDC4 expression is required for myoblast proliferation. However, the process of skeletal muscle regeneration in SDC4 KO mice is not well understood. Moreover, the molecular background of fusion-negative rhabdomyosarcomas is poorly understood. Thus, the following questions can be formulated:

1. Changes in the expression of myostatin and SDC4 during M. soleus regeneration?

2. What is the effect of SDC4 silencing on the levels of heparan sulfate proteoglycans and myostatin?

3. What is the effect of SDC4 silencing and SDC4/Rac1 pathways for the differentiation and fusion of myoblasts?

4. Does SDC4-mediated Rac1 activity affect MyoD expression, including PAK1 and cofilin activity during muscle differentiation?

5. What is the effect of SDC4 silencing on actin nanostructure and cortical actin during differentiation? Does it have any effect on the elasticity of C2C12 myoblasts?

6. Are there any alterations of SDC4 copy-number or RNA expression levels in human rhabdomyosarcoma?

3. MATERIALS AND METHODS

3.1. Cell culture and plasmids

C2C12 mouse myoblasts were stably transfected with plasmids expressing shRNAs (short hairpin RNAs) specific for SDC4. Non-transfected cells were cultured in 80% DMEM 20% fetal bovine serum and 50 μg/ml gentamicin. The transfected cells were selected in a medium containing 4 μg/ml puromycin. A medium containing 2% horse serum was used for differentiation. RD human rhabdomyosarcoma cells were maintained in 90% DMEM, 10% FBS and 50 μg/ml gentamicin.

3.2. Animal model

To induce regeneration of the soleus muscle of male Wistar rats (weighing 300–320 g), the snake venom notexin (from Notechis scutatus scutatus) was injected along the entire length of the muscle (20 μg notexin in 200 μL of 0.9% NaCl). The muscles were removed under anesthesia on days 0, 1, 3, 4, 5, 7, 10, and 14 after injury (n = 4 in each group). All animal experiments were conducted with approval obtained from the Animal Health Care and Control Institute, Csongrad County, Hungary.

3.3. QRT-PCR analysis

For qRT-PCR, total RNA was isolated from C2C12 cell lines and reverse transcribed (3 samples for each cell line). TaqMan probe sets [SDC1: Mm01275869_m1, SDC2: Mm04207492_m1, SDC3: Mm01179833_m1, SDC4: Mm00488527_m1, glypican-1 (Gpc1): Mm01290371_m1, perlecan (Hspg2): Mm01181173_g1, myostatin (Mstn): Mm00440328_m1, HPRT: Mm03024075_m1; all from ThermoFisher Scientific] and the

TaqMan Master Mix (Roche). Individual threshold cycle (Ct) values were normalized to the Ct values of HPRT. Relative gene expression levels are presented as log2 ratios.

3.4. Gel electrophoresis and immunoblotting

Cells were lysed in RIPA buffer, supplemented with 1 mM NaF and protease inhibitor cocktail. Soleus muscles were homogenized in a buffer containing 50 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1 mM NaF, 1 mM Na3VO4, and protease inhibitor cocktail and then centrifuged at 13,000 rpm for 5 min at 4°C to remove the pellet.

Protein concentration in the samples was determined using a BCA protein assay kit and equal amounts of proteins were resolved on polyacrylamide gel and transferred onto Protran membranes. Membranes were incubated with the primer mouse/rabbit antibodies, then incubation with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibodies, the peroxidase activity was visualized using the enhanced chemiluminescence procedure. Signal intensities were quantified using the QuantityOne software program.

3.5. Rac1 activation assay

Approximately 70%–80% of confluent cell cultures were lysed with Mg2+ lysis buffer and protease inhibitor cocktail. Then, the lysates were centrifuged (14,000 × g for 5 min at 4°C), the supernatant was aspirated, and then, the pellet was removed. For the detection of active Rac1-GTP, the Rac1 Activation Magnetic Beads Pull-down Assay was applied according to the manufacturer's instructions.

3.6. Rac1 GTPase inhibition

Rac1 activity was inhibited using NSC23766 trihydrochloride during myoblast differentiation. Cells were seeded into 6-well plates (1.8×10^5 cells/well) in growth medium and then shifted to a differentiation medium containing 50 μM NSC23766, and the medium was changed every 2 days.

3.7. Fluorescent, hematoxylin and eosin staining

For desmin immunostaining, myotubes were fixed with 4% paraformaldehyde on the 5th day of differentiation, and after 5-min permeabilization with 0.1% Triton X-100 in PBS, the samples were blocked in 0.1% bovine serum albumin (BSA) in PBS. For staining the differentiated myotubes, the samples were incubated overnight with mouse anti-desmin primary antibody at 4°C followed by incubation with anti-mouse Alexa Fluor 488-conjugated secondary antibody for 20 min. Nuclei were stained with Hoechst 33258.

For visualization of actin filaments, the myotubes were fixed with 4% paraformaldehyde and incubated with PBS containing 0.9% Triton X-100 and 4% BSA for 30 min. Then, the

samples were labeled with Alexa-647-conjugated phalloidin. Following nuclear staining with Hoechst 33258, the samples were immediately processed for dSTORM and confocal imaging.

Frozen sections (10 μm) of control and regenerating soleus muscles were fixed in acetone for 5 min and were stained by haematoxylin (0.1 %) and eosin (1 %).

3.8. Myotube analysis

Widefield fluorescence images of desmin- and Hoechst 33258-stained samples were acquired using a Nikon Eclipse Ni-U fluorescence microscope with a 10× objective lens (Nikon FI Plan Fluor 10×, DIC N2, NA = 0.30) and analyzed using the Digimizer image analysis software. The differentiation index was derived as the ratio of the number of desmin-positive cells and total number of nuclei. The value of fusion index was obtained by dividing the number of nuclei belonging to the desmine-positive myotubes with all counted nuclei. The area and length of each myotube were also quantified.

3.9. Confocal laser scanning microscopy

Confocal images were captured using a Nikon C2+ confocal scan head attached to a Nikon Eclipse Ti-E microscope. Confocal and superresolved dSTORM images were captured sequentially using the same microscope objective (Nikon CFI Apochromat TIRF, NA=1.49, X100) throughout the experiments to minimize spatial drift and reduce image registration issues. The setup and data acquisition process were controlled using the Nikon NIS-Elements 5.02 software, and the captured images were postprocessed in ImageJ-Fiji (https://fiji.sc/). The Nikon Laser Unit was used to set the wavelengths and the power of the applied lasers operated at 405 and 647 nm.

3.10. dSTORM measurements

Superresolution direct stochastic optical reconstruction microscopy (dSTORM) measurements were performed on a custom-made inverted microscope based on a Nikon Eclipse Ti-E frame. EPI-fluorescence illumination was applied at an excitation wavelength of 647 nm (2RU-VFL-P-300-647-B1, Pmax = 300 mW). The laser intensity was set to 2–4 kW/cm2 on the sample plane and controlled using an acousto-optic tunable filter. dSTORM experiments were conducted in a GLOX switching buffer, and the sample was mounted onto a microscope slide.

3.11. Cortical actin bundle width measurements

The localization information of the selected structures was exported by the rainSTORM program using the “Export box section” tool into the IFM Analyzer code written in MATLAB R2018b. The IFM Analyzer code was originally developed for the quantitative evaluation of dSTORM images on Indirect Flight Muscle Sarcomeres. The same code was used in the present

study to retrieve the epitope distribution information from raw localization data and determine the width of the cortical actin bundles.

3.12. Skeletonization

An additional MATLAB code was written to skeletonize the superresolution images and determine the number and length of branches of the actin filaments. First, the images were binarized with a threshold gain of Otsu's method or with a threshold set manually through ImageJ-Fiji. The images were filtered with a 2D Gaussian smoothing kernel with a standard deviation of 3–4 pixels (60–80 nm) to homogenize the pixelated images and were again binarized using the Otsu's method. Built-in MATLAB functions (bwskel) were used to skeletonize the binary images and to calculate the branch numbers and branch lengths (bwmorph and bwdistgeodesic). Short branches were omitted from the calculation (the minimum branch size was set to 120 nm).

3.13. Atomic force microscopy

Cells (all types) were cultured on the surface of a glass coverslip. After medium change, the coverslips were mounted into the heating chamber of the microscope in a standard glass-bottomed plastic Petri dish and maintained at 37°C during measurements. Elastic maps were recorded using an NTegra Spectra atomic force microscope running the Nova Px 3.4.1 driving software, mounted on the top of an IX73 inverted optical microscope to facilitate initial positioning. Each cantilever was calibrated before the experiments based on the Sader method. Elastic parameters were calculated using the Hertz model with the assistance of the driving software.

3.14. Rhabdomyosarcoma cases and genomic datasets

Genomic data from 199 specimens, collected from 199 patients and deidentified before use, were compiled from the following three dataset sources: the National Cancer Institute, the Children's Oncology Group, and the University of Texas Southwestern (UTSW). Genomics analyses of archived patient samples were conducted at the UTSW Medical Center with the approval of its institutional review board (STU 102011-034). The original genomic data is deposited to dbGAP database with accession number phs000720.

3.15. Genomic sequencing, copy number, and gene expression data analysis

Whole-genome and whole-exome sequencing reads were aligned to the human reference genome (hg19), and somatic protein-altering mutations were identified using the Genome Analysis Tool Kit pipeline. SNP arrays were processed using the SNP-FASST segmentation algorithm implemented in the Nexus BioDiscovery software. Significantly altered CNVs were examined using the GISTIC method using a default q value of 0.25 to define

statistical significance. For gene expression data, RNA was processed using the Affymetrix Exon 1.0 ST array platform according to the manufacturer's recommendations. CEL files were analyzed using R/BioConductor with robust multiarray average normalization and custom PERL scripts.

3.16. Statistical analysis

Statistical analysis was conducted using the GraphPad Prism 6 software, Student's t-test and one-way ANOVA, and a posthoc test (Sidak and Newman-Keuls) for peer pair comparison. All evaluated data were expressed as average + SEM. $p < 0.05$ denoted statistical significance.

4. RESULTS

4.1. The expression of SDC4 and myostatin during *in vivo* myoblast differentiation

Muscle regeneration can be artificially induced by injecting the snake venom notexin. It rapidly induces myonecrosis and, because it does not affect satellite cells, a subsequent regeneration of the tissue occurs. Western blot experiments showed a transient upregulation of SDC4 expression during the proliferation phase, and simultaneous a low level of mature myostatin and high level of promyostatin.

4.2. SDC4 knockdown influences the levels of heparane sulfate proteoglycans and myostatin

Silencing of SDC4 upregulated the levels of SDC3 and SDC1, and slightly increased the amount of SDC2 transcripts. The heparan sulfate proteoglycan glypican-1 and perlecan showed weak upregulation following SDC4 silencing. The level of myostatin mRNA increased in SDC4 knockdown cells, which was significant in shSDC4#1 cell line.

4.3. SDC4 knockdown increases myoblast differentiation and fusion *in vitro*

An excellent in vitro model exists to study muscle differentiation, since shifting mouse C2C12 myoblasts from growth medium to low-serum fusion medium induces the formation of multinucleated, myosin expressing myotubes. The expression of SDC4 gradually decreased during the 5-day differentiation of C2C12 murine myoblasts, and the proliferating myoblasts showed higher SDC4 levels, whereas the differentiated myotubes showed lower SDC4 levels.

The expression of Myf5 showed a peak at day 1, whereas those of MyoD and desmin continuously increased, indicating the appropriate differentiation of the samples.

Representative immunoblots showed that both MyoD and MyoG expression increased earlier in SDC4 silenced cells during differentiation. Desmin-stained representative images depicted differences in the number and shape of myotubes after silencing SDC4 expression, wherein SDC4-knockdown cells formed much longer and bulkier myotubes than those of control cell lines. We found significant increases in the differentiation index and fusion index in both SDC4 silenced cell lines. Nuclear number analysis revealed that the number of nuclei in the myotubes increased significantly after SDC4 knockdown. The majority of SDC4 silenced myotubes contained 3–5 or > 5 nuclei, whereas control cell lines contained primarily 2 nuclei per myotube, suggesting that SDC4 knockdown is involved in myonuclear accretion to promote myotube formation. Moreover, both the area and length of myotubes were larger in SDC4 silenced cell lines.

4.4. Rac1 activity is required for increased fusion of SDC4-knockdown cells

Our results indicated that silencing SDC4 expression increased the amount of Rac1-GTP. We observed that both the phospho-PAK1(Thr423)/PAK1 and phospho-cofilin(Ser3)/cofilin ratios were elevated in SDC4 knockdown cells. As SDC4 knockdown increased the Rac1-GTP level and the phosphorylation of PAK1 and cofilin, we next tested the effect of Rac1 inhibition on myoblast differentiation after silencing SDC4 expression. Representative, desmin-stained widefield fluorescence images depicted that NSC23766 treatment inhibited myotube formation in either control or silenced cells, although desmin was expressed. Moreover, NSC23766 administration abrogated the increases in MyoD expression and also the increases in pPAK1(Thr423)/PAK1 and phospho-cofilin(Ser3)/cofilin ratios in SDC4 silenced cells.

4.5. The levels of Tiam1, phospho-PAK1, and phospho-cofilin are gradually reduced during in vitro and in vivo myogenesis

During the 8-day differentiation period of C2C12 cells, the high Tiam1 level continuously decreased after the 5th day. We also evaluated the amounts of Rac1-effector phospho-PAK1 and phospho-cofilin and observed that during the early stages of differentiation, from day 2 onward, an intense increase occurred followed by a decrease from day 5 in phospho-PAK1 (Thr423) levels. Consistent with phospho-PAK1 levels, the levels of phospho-cofilin(Ser3) exhibited the same pattern. During both in vitro differentiation and in vivo skeletal

muscle regeneration, the levels of Rac1 activator Tiam1 and the phosphorylation of the Rac1-effector PAK1 and cofilin were transiently increased. These increases can result in an intense remodeling of the actin network during the formation of myotubes.

4.6. Silencing SDC4 expression affects the nanoscale structure of the actin network by increasing cortical actin thickness and number of branches

In this study, we showed that SDC4 affects the activity of Rac1 in myoblasts, a key regulator of actin remodeling. Considering these important roles of SDC4 in actin cytoskeleton organization, we monitored the changes in the actin nanostructure during differentiation after silencing SDC4 expression. SDC4 silenced cell lines exhibited a significantly thicker cortical actin network than that of the control cells during differentiation, and the evaluation indicated an approximately 50% broadening of the silenced cell lines compared to that of the non-transfected and scrambled cell lines.

For the nanoscale analysis of the branched structure of the actin network, the dSTORM images of 3-day-old mononuclear differentiated but not yet fused myoblasts were pixelizated and converted into binary images. The analysis revealed an increase in the number of branches and normalized branch number in SDC4 knockdown cells. However, the average length of the individual branches was shorter compared to that of control cells. These changes of the actin cytoskeleton can result in a more compact actin network that promotes fusion of the SDC4 silenced cells.

According to our results, the serum content of cell culture media (20% FBS vs. 2% horse serum) affected the actin nanostructure of C2C12 cells. Reducing the serum content the length of the individual branches of the actin cytoskeleton decreased in all cell lines. SDC4 silencing also decreased the the length of branches independently of serum content. The high serum content resulted in less brances of the actin nanoctructure in SDC4 silenced cells, whilst the number of branches of the silenced cells increased in the serum-reduced medium compared to controls.

4.7. Silencing SDC4 expression reduces the elasticity of myotubes

Given the role of SDC4 in actin cytoskeleton remodeling, we hypothesized that SDC4 can affect the elasticity of cells. Therefore, we next examined how the elasticity of cells changes during fusion after silencing the expression of SDC4. Therefore, silencing SDC4 expression decreases cell elasticity, i.e., these cells are tougher than control cells in accordance with the observed alterations in the cytoskeletal structure.

4.8. Copy-number amplification and increased expression of SDC4 in human rhabdomyosarcomas

Based on our present study on the role of SDC4 in myoblast differentiation and considering the unknown role of SDC4 in rhabdomyosarcoma, we investigated the presence of SDC4 copy number amplification and loss events in human rhabdomyosarcoma samples. According to copy number analysis, SDC4 was highly amplified in rhabdomyosarcomas, especially in FNRMSs, as genomic analyses revealed copy number amplification events in 28% of fusion-negative tumors. Among 49 FPRMS patients, 6 showed gain of SDC4, but none showed loss of SDC4; however, among 150 FNRMS cases, 42 showed gain of SDC4, and 1 showed loss of SDC4. Based on the mRNA sequencing data, FNRMS cases were accompanied by increased SDC4 mRNA expression compared to that in FPRMS cases, suggesting SDC4 as a potential tumor driver gene in FNRMS promoting tumorgenesis.

The observed high SDC4 expression in RD cells is consistent with the copy-number amplification and high mRNA expression of SDC4 in FNRMS tumors.

5. DISCUSSION AND CONCLUSION

During regeneration following skeletal muscle injury, quiescent satellite stem cells are activated, they proliferate, differentiate into myoblasts and then fusion into multinucleated tubular myotubes. It is known from the literature that skeletal muscle morphology and regeneration are impaired in SDC4 transmembrane proteoglycan gene knockout mice, but it is unclear how the absence of SDC4 leads to impaired muscle regeneration. Furthermore, the molecular background of fusion-negative rhabdomyosarcomas is poorly understood.

The novel findings of the present thesis can be summarised as follows:

1. We observed that SDC4 expression was increased early in regeneration. The dominant myostatin form during regeneration was immature promyostatin, which showed the highest expression on day 4 of regeneration.

2. We showed that silencing of SDC4 upregulated the levels of SDC3 and SDC1 and slightly increased the amount of SDC2 transcripts in C2C12 cells. The heparan sulfate

proteoglycan glypican-1 and perlecan showed weak upregulation following SDC4 silencing. The level of myostatin mRNA increased in SDC4 knockdown cells.

3. SDC4 silencing increased the differentiation and fusion of myoblasts. The SDC4-knockdown cells formed much longer and bulkier myotubes than those of control cell lines. The number of nuclei in the myotubes increased significantly after SDC4 knockdown.

4. Rac1 activity is required for increased fusion of SDC4-knockdown cells. During muscle differentiation, the gradually decreasing expression of syndecan-4 allows the activation of Rac1, thereby mediating myoblast fusion.

   Rac1 inhibition (with NSC23766) abrogated myotube formation in both control and silenced cells. Also, the increase in MyoD expression was abolished, as well as the increase in phospho-PAK1(Thr423)/PAK1 and phospho-cofilin(Ser3)/cofilin ratios in SDC4 silenced cells.

5. Silencing SDC4 expression increased the amount of Rac1-GTP in myoblasts and both the phospho-PAK1(Thr423)/PAK1 and phospho-cofilin(Ser3)/cofilin ratios were elevated.

6. The levels of Tiam1, phospho-PAK1, and phospho-cofilin are gradually reduced during in vitro and in vivo myogenesis.

7. Silencing SDC4 expression affects the nanoscale structure of the actin network by increasing cortical actin thickness and number of branches and reduces the elasticity of myotubes. This may explain the increased fusion capacity of SDC4 silenced cells and thus role in providing the mechanical basis for fusion.

8. SDC4 copy-number amplification was observed in 28% of human fusion-negative rhabdomyosarcoma tumors and was accompanied by increased SDC4 expression based on RNA sequencing data. Our study suggests that syndecan-4 can serve as a tumor driver gene in promoting rhabdomyosarcoma tumor development.

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