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The role of the cytoskeletal septin7 protein in skeletal muscle function and regeneration

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1. Introduction

As medical science evolves, we strive to understand how different organ systems work in order to preserve quality of life. The proper functioning of the striated skeletal muscle is of paramount importance for health and economy, both at an individual and societal level. Muscle atrophy, muscle loss in old age and muscle deterioration due to microgravity are all limiting factors of our time.

The previously known cytoskeletal constituents - actin, intermediate filaments and microtubules - have been studied and their role partially elucidated, but in recent decades a new family of skeletal proteins, the septins, has been identified. These septin proteins have been shown to be able to form oligomers and higher structures with each other. They form macromolecular complexes and are involved in cell division, differentiation, migration, immune responses and metastasis. However, the role of septins in skeletal muscle function is still unclear. Based on their structural composition, we can classify septins into four major groups. Septin7 is the only one in its group and, as far as we know, is extremely important in the assembly of higher complexes.

Their *in vitro* and *in vivo* roles have been described for the first time by our group and this knowledge may provide new insights into the molecular regulation of striated skeletal muscle fibres. In this paper, we present the results of *in vivo* and *in vitro* experiments in mice.

2. Literature review

2.1 The cytoskeletal system

The cytoskeleton, also known as the cytoskeleton, is a complex and dynamic network of interacting and interconnected proteins present in the cytoplasm of all cells. It consists of three main components: microfilaments, intermediate filaments and microtubules. The primary function of the cytoskeleton is to regulate cell shape, resist deformation, and to provide connection to the extracellular space and the connective tissue and other cells present there. The cytoskeleton is also involved in cell migration because of the properties mentioned above. In addition, it is involved in several signalling pathways, in the uptake of extracellular material, i.e. endocytosis, in the segregation of chromosomes during cell division, in the cytokinesis phase of

cell division and in the organisation of cell division In addition to the cellular processes mentioned above, it can also build special structures such as flagella, cilia, lamellipodia and podosomes.

2.1.2 Actin

The actin protein is globular in structure, approximately 42 kDa in size and 4-7 nm in diameter. They form microfilaments in the cytoskeleton. Three main isoforms of actin have been identified: alpha, beta and gamma. In cells, it can appear as a free monomer, G-actin, or as a linear polymer, i.e. microfilament, in the form of F-actin. They are essential in many vital cellular functions such as migration, cell division, cell shape determination, muscle contraction, transport of vesicles and organelles, various signalling processes and the formation of cell-cell and cell-environment contacts. In these processes, the interactions of actin proteins with the cell membrane play a key role. They are involved in the assembly of desmosomes and haemidesmosomes.

2.1.3 Tubulin

Tubulins are evolutionarily highly conserved proteins that are found in all eukaryotes.They have GTPase activity, the ability to assemble and disassemble, and can form polymeric structures. Both α and β tubulins have a mass of about 50 kDa. The αβ-tubulin dimers assemble into microtubules, a dynamic polymer that is involved in many functions, but their primary role is in mitosis. They are essential for the division of chromosomes to form microtubules associated with the kinetochore region and the division spindle. They are also essential for the organisation of intracellular vesicle transport.

2.1.4 Intermediate filaments

Intermediate filaments get their name from their diameter (about 10 nm), which is between the diameter of actin filaments (about 7 nm) and microtubules (about 25 nm). Based on observations so far, they appear to play a fundamental structural role by providing mechanical strength to cells and tissues. The tight junctions between non-polarized protein units provide filaments with high tensile strength. This makes them the most stable component of the cytoskeleton. This

resistance is the reason for their occurrence in particularly durable structures such as hair, skin and nails. The primary function of intermediate filaments is to establish cellular connections and prevent rupture of epithelial cell layers under tension. Intermediate filaments increase the resistance of cells and tissues to torsional, compressive, stretching and bending forces. They also participate in the formation of the nuclear membrane, where they help protect and organise the cell's DNA content.

2.2 Septins

2.2.1 The septin protein family

The name septin derives from the term separation and was first described in the budding yeast *Saccharomyces cerevisiae*, where it plays an important role in cell division, the separation of progeny cells. Septins are evolutionarily highly conserved guanosine triphosphate (GTP)-binding proteins and also have GTPase function. In addition to their earliest recognised role in cell division, septins may have other cell-specific functions. Hence, they play important roles in many physiological and pathological cellular processes, such as carcinogenesis, exocytosis, endocytosis and membrane organisation. To date, 13 human septin isoforms have been identified and classified into four functional groups (SEPT2, SEPT3, SEPT6, SEPT7) based on structure and sequence homology.

2.2.2 Structure and function of septins

By examining the function of the septins and the amino acid structure required for their function, we can observe that an AKAD sequence and a Walker A (GxxxxxxGKS/T) and B (DxxG) motif responsible for GTP-specific binding (polybasic region) are present in the septins. Surprisingly, the septins are able to oligomerize through their GTP-binding domains. Formation of these complexes requires interactions between the adjacent GTP-binding domains, i.e. the G surface, and the NC surface formed by the N- and C-terminal stretches.

An important structural feature in the structure of septins is the sequence called Septin Unique Element (SUE), which extends from the G to the NC surface and allows the formation of filamentous structures.

Coiled coil (CC) sequences are found in the C-terminal domain of a significant proportion of septin isoforms. In addition to the CC domain, two other structural parts are found within the C domain: C_N and the region following CC. The

C_N region has a high degree of structural and sequence variability and, as a result, shows extreme flexibility. The studies show that the region accompanying the coiled coil domain is disordered in structure. The C-terminal region is a polybasic sequence. This motif, in combination with various polybasic domains (PB1 and PB2) found in other septins, may help to form a number of membrane-septin interactions.

Previous studies in yeast and *Drosophila* have shown that several septin isoforms can be involved in the assembly of a molecular complex. As we know Septin7 is the only representative of its structural group. It plays an essential role in the formation of hetero-oligomeric complexes and in the assembly of higher order cytoskeletal structures.

2.3 The septin7 protein

The gene sequence encoding the septin7 protein (SEPTIN7) in humans is located on chromosome 7p14.2 and contains 1254-1311 nucleotides, with 19 exon regions encoding 418-437 amino acids. Septin7 can form a dimer with itself via a G-site.

2.3.1 The role of the septin7 protein in physiological processes

The importance of septin7 has been described in a variety of cellular processes. In experiments using the Drosophila model system, both the absence and elevated expression of septin7 resulted in flight abnormalities, suggesting involvement of calcium stores and calcium homeostasis. These results suggest that septin7 may be involved in the regulation of intracellular Ca^{2+} levels either directly or indirectly. The role of the septin system and septin7 in migration and phagocytosis is an important factor for the function of different immune cells. The role of septin7 has been investigated in neurons and the nervous system, where it has been detected in several cell types. Because of its role in cell division, it may influence the development of certain gliomas, papillary thyroid cancer and liver carcinomas

Excessive levels of septin7, which may be caused by increased expression or decreased degradation, may inhibit cell proliferation and arrest the cell cycle and induce apoptosis of tumour cells. This apparently contradictory effect of septin7 may be due to its involvement in two distinct process of cell division.

Looking at the following examples, it can be stated that the role of septin7 may be various in different cells. This diversity may depend, among other things, on the cell-specific environment and on the partner proteins expressed in the cell types.

2.4 Characterisation of skeletal muscle fibres

2.4.1 The structure of skeletal muscle fibres

The individual skeletal muscle fibres are surrounded by a special cell membrane, the sarcolemma. The cell's cytoplasm contains contractile proteins, which make up a significant mass of the cell's protein content. The contractile system is made up of two main components: the thick filament is made up of myosin, while the thin filament is made up of actin molecules. The myofilaments are arranged in myofibrils surrounded by an advanced membrane system, the sarcoplasmic reticulum (SR). A muscle fibre is built up by a few hundred myofibrils, each of which can be broken down by the Z-plates into several sarcomeres of approximately 1.9-2.1 μm in length.

2.4.2 The contractile proteins

Actin and myosin are responsible for the formation of contractions. During contractions, interactions between these two proteins at the molecular level create the basis of movement essential for life.

Myosin is a motor protein found in skeletal muscle that is involved in the energy-intensive conformational changes responsible for muscle contraction The myosin molecule has a bifurcating head region with ATPase activity that is linked to a cervical segment. Hundreds of myosin molecules are assembled into a single complex that forms the thick filament.

The main component of the thin filament is actin in skeletal muscle. Several proteins involved in the regulation of contraction are associated with actin. Tropomyosin, which is also filamentous, is wrapped around the actin filament in a conformation in which the tropomyosin molecule covers the myosin-binding site of the actin monomer. The role of tropomyosin is to inhibit the formation of actin-myosin contacts and thereby regulate contraction. A troponin complex, consisting of three subunits, is also attached to actin. Troponin-C (TnC, MW=18 kDa) plays a role in Ca^{2+} ion binding; troponin-T (Tn-T, MW=37 kDa) is a coupling protein that anchors the troponin complex to tropomyosin; troponin-I (Tn-I, MW=24 kDa) is directly responsible for the inhibitory effect by preventing cross-bridging between actin and miosin. During the electromechanical coupling, the elevated Ca^{2+} levels cause the complex to bind Ca^{2+} and change conformation, which results in the loss of the inhibitory structure of TnI and the formation of actin-myosin contacts.

2.4.3 T-tubule system and sarcoplasmic reticulum

For proper diffusion movement in the sarcolemma, there are transverse channels in the fibre towards the inside, which form a special system of channels called the T-tubule system. Closely related to the T-tubule system is the sarcoplasmic reticulum (SR), which is a modified endoplasmic reticulum present in skeletal muscle fibres. The T-tubules are joined to the SR by a sprouting compartment, the terminal cisterna, on either side, and the three tubes together form the triad structure. The sarcolemmal dihydropyridine receptor (DHPR), sensing an action potential, changes its conformation and induces a conformational change in the type 1 ryanodine receptor (RyR1), which is in direct contact with the DHPR. Ca^{2+} released from the SR intracellular Ca^{2+} store via the open RyR1 allows actin-myosin contact to form and initiate contraction. This process, when the electrical impulse (action potential) is converted into contraction, is called electromechanical coupling, also known as EC coupling.

2.5 *In vivo* muscle development (myogenesis) and regeneration

Three main steps need to take place for muscle fibres to form. The initial step in the formation of muscle cells is the activation and division of muscle stem cells also known as satellite cells (SC). The resulting muscle cells, after preliminary differentiation, fuse together in the second phase of development to form the characteristic multinucleated muscle fibres. In the last stage, the muscle fibres, which are still immature, differentiate into the corresponding skeletal muscle fibre type.

In addition to myogenesis during development, there is also a need to replace/repair lost/damaged fibres during muscle injury. This complex process is called regeneration. It can be divided into five overlapping phases, with the phases following each other in a strict sequence.

The first phase is skeletal muscle injury itself and subsequent necrosis. Depending on the extent of the tissue damage and the time of exposure, we can speak of micro-injuries, fibre damage and complete destruction of muscle tissue.

The process following necrosis is the inflammation caused by the injury. This process involves the cells of the immune system, more specifically its natural immunity side. Neutrophil granulocytes and macrophages are activated in response to damage associated molecular pathways (DAMPs) and inflammatory mediators released from

the dying muscle fibres. TNF-α (tumor necrosis factor alpha), IFN-γ (interferon-γ), IL-1β (interleukin-1β), IL-1, IL-8, IL-6) activate stem cells necessary for regeneration.

The previously mentioned skeletal muscle-specific stem cells are the satellite cells, which are responsible for the regenerative myogenesis phase. Non-randomly present between muscle fibres, the satellite cells are responsible for maintaining their surrounding space, also known as niche. The most important activation markers are Pax7, Mcad, VCAM1, and also several myogenic markers such as desmin, myogenic factor 5 (Myf-5) and muscle cell commitment protein (MyoD).

Muscle cells that develop from satellite cells and incorporate into newly forming fibres undergo differentiation and maturation. During this phase, due to increased protein synthesis, the nuclei, which are normally peripherally located, occupy a central position in the fibre to facilitate protein synthesis and transport. The extracellular matrix (ECM) around the fibre is also synthesised during this phase, which is why it is called tissue remodelling. The ECM contains a diverse composition of proteins, proteoglycans and glycoproteins, which play an important role as structural support, in force transmission and in the regulation of cell division and differentiation.

The final phase of regeneration is the innervation of mature muscle fibres by motoneurons. For this the neuromuscular junction (NMJ) must be re-established. In this specialised chemical synapse, proper signal transduction requires clustering of muscle-type nicotinic acetylcholine receptors (nAchR). The process of clustering is currently not fully understood, but interactions between the cytoskeleton and the sarcolemma may play an important role. The detection of newly formed NMJs is confirmed by the return of muscle strength.

3. Statement of the problem and objective

Our knowledge suggests that septin proteins may play an important role in many molecular processes in skeletal muscle fibres, but their significance is being far from fully understood. Mapping the roles of septins, and in particular of septin7, may help to answer a number of currently open questions concerning skeletal muscle function. Our group has set out to investigate in detail the role of septin7 in skeletal muscle function, including the construction of an *in vivo* mouse model.

In this paper, I present the results obtained *in vivo* in a genetically modified, induced septin7 knock down mouse strain, which are related to muscle function and regenerative capacity. In addition, a series of experiments will be discussed performed *in vitro* on samples from the same mouse strains, involving molecular biological, morphological and muscle strength measurements.

In our experiments we planned to answer the following questions:

- To what extent are septins detectable in mice and human muscle samples? Does the level of expression vary during the development of mice?
- What is the role of septin7 in skeletal muscle based on a comparison of a control and a skeletal muscle-specific septin7 knock down mouse strain?
- What role might septin7 play in muscle force generation?
- What effect does septin7 have on the regeneration process?

4 Methods

4.1 Analysis of human samples

The human skeletal muscle samples were used as described in the Scientific and Research Ethics Committee Regulation (EC) No 7917-1/2013/EU 113/2013. Muscle samples from *quadriceps femoris* of patients who underwent amputation were used in the studies. Limb removal surgery and muscle tissue sampling were performed at the University of Debrecen, Kenézy Gyula Teaching Hospital.

4.2 Animal experiments and the keeping of laboratory animals

The animal experiments were carried out in accordance with the European Union Regulation 86/609/EEC and the University of Debrecen's Workplace Animal Welfare Committee Regulation 2/2019/DEMAB. The mice were kept in plastic cages with lattice roofs and had free access to mouse food and drinking water. Their housing lighting was automated with 12-hour dark and light cycles and the room temperature was set between 22-25°C.

4.3 Tamoxifen feeding

Oral Tamoxifen feeding was started at 4 weeks of age, immediately after weaning from the mother in all groups of animals. The animals used in the experiments were fed continuously with the Tamoxifen-containing diet (Envigo, TD 130857) for 3 months. The dose of the diet was 500 mg Tamoxifen per kg of diet, so that the daily intake of Tamoxifen was 80 mg/kg body weight (kg) per day at a daily consumption of 3-4 g per day for an average body weight of 20-25 g of mice.

4.4 Establishment of the HSA-MCM transgenic mouse strain

B6.Cg-Tg(ACTA1-cre)79Jme/J transgenic mouse strain was purchased from the Jackson Laboratory (Bar Harbor, ME). A segment encoding the Cre recombinase gene combined with the human skeletal muscle actin (ACTA1) promoter is present in HSA-Cre transgenic mice, and therefore expression of the Cre enzyme occurs only in adult and differentiated striated skeletal muscle fibers. The HSA-MerCreMer (HSA-MCM) gene sequence also contains a Cre recombinase (Cre) enzyme and a modified estrogen binding domain (ERT2), thus Cre enzyme expression can be induced in the presence of tamoxifen. By crossing the HAS-MCM strain with a strain containing the gene bordered by loxP sequences, we can create a skeletal muscle-specific inducible deletion. Genomic DNA was isolated from B6.Cg-Tg(ACTA1-cre)79Jme/J mouse strain from the fingertips of the animals and were PCR-tested for the presence of the HSA-MCM transgene.

4.5 Verification of Septin7 deletion by genotyping

The mouse strain C57BL/6J Septin7flox/flox (SS00) was obtained from the laboratory of Prof. Dr. Matthias Gaestel, Institute of Physiological Chemistry, Hannover Medical University. These mice were crossed with B6.Cg-Tg(ACTA1-cre)79Jme/J (ssC0) mice. The resulting offspring (SsC0) were backcrossed with SS00 genotypes. Litters from these breedings were used for Tamoxifen feeding in the experiments. The offsprings included individuals whose genomes contained flox sequences at the boundary of exon 4 of the septin7 gene and the gene sequence also encoded the enzyme Cre. To verify the extent of the septin7 modification, skeletal muscle samples *(m. quadriceps femoris, m. biceps femoris* and *m. pectoralis*) were collected from Cre+septin7flox/flox mice (hereafter Cre+) and genomic DNA was

isolated. Littermates that did not contain the MerCreMer construct were considered as control samples. PCR primers designed at the deletion site were used to separate flox sequence-containing and wild-type or control samples.

4.6 *In vivo* experiments

4.6.1 *In vivo* CT experiment

For *in vivo* imaging, mice were anaesthetised with 3% isofuran using a device designed for anaesthetising small animals. A nanoScan SPECT/CT (Mediso Ltd, Hungary) was used to acquire whole-body CT scans with the following settings: X-ray tube voltage 60 kVp, current 86 mA; exposure time 170 ms per image; voxel size 1 × 1 mm. CT images were acquired and analyzed using Nucline and InterView FUSION software (Mediso Ltd, Hungary).

4.6.2 Voluntary run in a treadmill

Mice in different groups were kept in cages equipped with a treadmill (Campden Instruments Ltd., Loughborough, UK) to test voluntary running. The treadmills, connected to a computer, measured the speed of the device at 20-minute intervals and thus the voluntary activity of the mice for 14 days. The average and maximum running speed and distance per day and the running time were determined individually. These results were averaged across study groups.

4.6.3 Measurement of forelimb strength

The grip strength of the forelimb was measured using a specially designed measuring system. The animal is held painlessly by the base of the tail on a metal wire that is convenient for the mouse to grip. The animal grips the wire and the pulling force is measured continuously and the subject is moved backwards until the maximum force is reached, i.e. until the wire is released. The movement of the wire is detected by a force sensor connected to a computer. The test was performed 10 to 15 times per animal and averaged over these measurements to obtain a single measurement point. The grip force was always measured one day before the animals were sacrificed.

4.7 *In vitro* experiments

4.7.1 Measurement of muscle strength

After dissection of fast and slow muscle types, i.e. *m. extensor digitorum longus* (EDL) and *m. soleus* (Sol), the muscles were tied at the end of tendons and placed in a special measuring chamber. In the measuring chamber, the muscles were fixed at one end while the other end was attached to the arm of the force sensor. The experiment was performed under continuous solution flow (10 ml/min) in Krebs solution (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM HEPES, 10 mM glucose, 10 mM $NaHCO_3$; pH 7.2; 95% O_2 and 5% CO_2 at room temperature). Muscle movement was detected by a capacitive mechano-electric force transducer (Experimetria, Budapest, Hungary). To elicit individual twitches, a super-maximum pulse of 2 ms duration was induced between two platinum electrodes placed around the muscle. The force generation recorded as a result of the stimulation was measured on a TL-1 DMA instrument at a frequency of 2 kHz, and the data were evaluated using Axotape software (Axon Instruments, Foster City, California, USA). The appropriate muscle length was set to generate maximum force, after which the muscles were left at the appropriate sarcomere length for 6 min to adjust to a new equilibrium state. Individual twitches were analysed at a stimulation frequency of 0.5 Hz, where at least 10 individual twitches were analysed for each muscle. Within each twitch series, the amplitude of individual cycles varied by less than 3%, so the average of the amplitude of individual twitches was chosen to characterize each muscle. In the tetanus test, muscles were stimulated with a 200 ms long pulse with 200 Hz frequecy for EDL and a 500 ms long pulse with 100 Hz frequency for Sol. To determine the duration of twitching and tetanus, the duration between full contraction and 10% relaxation was used.

4.7.2 Isolation of individual muscle fibre

Individual muscle fibres were obtained from *M. flexor digitorum brevis* (FDB) muscle by enzymatic digestion. FDB muscle was digested in calcium-free medium containing 0.2% type 1 collagenase (Sigma) at 37°C for a time corresponding to the weight of the mouse. By lightly titurating the muscle in Tyrode's solution (1.8 mM CaCl2, 0 mM EGTA), individual intact fibers were obtained. These individual fibres can be stored for 2 days at 4°C in medium on a glass cover slip.

4.8 Immunochemical staining of individual muscle fibres and cryostat or paraffin sections

For immunochemical analysis, samples were first fixed in 4% paraformaldehyde (PFA) for 20 min. The treatment of cryostat slides used for immunohistochemistry was identical to that of the immunocytology treated slides, the only difference being that frozen sections were allowed to thaw for 15 min before fixation. During and after 20 minutes of fixation, the sample retains its original structure. Fixation was followed by neutralization of the uncrosslinked PFA using 100 mM glycine-PBS solution. For the delivery of antibodies into the cell, permeabilization of the cell membrane with 0.5 % Triton-X (Sigma) PBS solution for 10 min was required. The samples were then washed three times for 10 minutes with PBS solution. To cover non-specific binding sites, Serum-free Protein Blocking Solution (Dako, Los Altos, CA) was used for 30 min. Primary antibodies (anti-RyR1, anti-Septin7, anti-CD45 and skeletal muscle specific anti-α-actinin) were incubated overnight at 4°C in a humidity chamber on the samples. Non-bound antibodies were removed by washing with PBS solution. A fluorophore-conjugated secondary antibody was added against the heavy chain of the primary antibody. After removal of unbound antibodies by further washing, the sections were covered. Samples containing Alexa Fluor 488, TRITC and DAPI fluorophores were imaged with an AiryScan 880 laser scanning confocal microscope (Zeiss, Oberkocken, Germany) using a 20x and 40x air objectives and 63x oil immersion objective. The aforementioned fluorophores were excited at wavelengths of 488, 543 and 405 nm and emission was detected in the wavelength ranges 520-550 nm, 560-580 nm and 420-490 nm, respectively (in the order listed).

Paraffin sections had to be deparaffinized before treatment. Although these sections were already fixed, they were post-fixed as described above. Endogenous peroxidase enzymes were inhibited with a 0,5 % hydrogen peroxide (H_2O_2) solution. As secondary antibodies antibodies bound with peroxidase enzymes were added to the paraffin sections. After treatment with DAB reagent, a brown precipitate is formed at the site of the antibody complexes. The sections were covered after dehydration and examined by light microscopy.

4.9 Muscle regeneration studies

Skeletal muscle injury was induced by injecting $BaCl_2$ into the muscle. Injection of 20 µl of 1.2% $BaCl_2$ dissolved in physiological saline solution into the *tibialis anterior* (TA) muscle caused sterile inflammation. One leg of each mouse was

used as control and was injected with physiological saline solution. Individuals in the study groups were sacrificed on days 4 and 14 after injection. TA samples from both injected and non-injected, i.e. control, legs were subsequently subjected to Western blot analysis and preparation of cryostat and paraffin sections. Histological studies were performed using Hematoxylin-Eosin (HE) and DAB staining.

4.10 Electron microscope sample preparation and processing

Freshly isolated TA muscles were fixed *in situ* in 3% glutaraldehyde Millonig buffer. The detached smaller muscle bundles were post-fixed in 1 percent OsO_4 aqueous solution. After rapid dehydration via ethanol gradient, propylene oxide treatment was followed by Durcupan (Sigma) epoxy resin embedding. Ultrathin longitudinal and transverse sections were made using a Leica Ultracut UCT (Leica Microsystems, Vienna, Austria) ultramicrotome. The sections were stained with uranyl acetate and lead citrate. The sections were imaged using a JEM1010 transmission electron microscope (JEOL, Tokyo, Japan). The EM images were analyzed using ImageJ software (NIH, Bethesda, MD) and the area, circumference, axial ratios, and circularity of individual myofibrils and mitochondria were determined from longitudinal and cross-sectional images of Cre- and Cre+ mouse samples.

4.10 RNA isolation, Real-time PCR and quantitative PCR analysis

Human muscle biopsy samples and mouse muscle samples were homogenized in 4 Trizol (Molecular Research Center, Cincinnati, OH, USA) and mRNA was extracted using an organic solvent RNA isolation method. After addition of 20% chloroform, lysates were centrifuged at 16 000 g for 15 min at 4°C. The supernatants of the samples were precipitated with 500 µl RNase-free isopropanol at room temperature for 10 min. After centrifugation at 12 000 g, the precipitated RNA was washed with 75 % ethanol and centrifuged again. The precipitated RNA was re-dissolved in RNase-free water and the RNA concentration and purity were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and the samples of known concentration were stored at -80°C until use. To perform the reverse transcriptase (RT) reaction, the reaction mixture (per 20 µl total volume) contained 1 µg RNA, 0.25 µl RNase inhibitor, 0.25 µl oligo (dT), 2 µl dNTP (200 µM) in RT buffer. Amplification of specific cDNA sequences was performed using predesigned specific primer pairs from Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA)

purchased from Bio Basic (Toronto, Canada). The specificity and applicability of the custom-designed primer pairs were verified in silico using the NCBI Primer-BLAST online database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Sequences of interest were amplified using a programmable PCR instrument (Labnet MultiGene 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ). An initial denaturation was performed at 94°C for 1 min, followed by 30 cycles with a denaturation step at 94°C (30 s); then annealing at optimized temperature for each primer pair for 30 s; finally, extension was performed at 72°C for 60 s, followed by final elongation at 72°C for 5 min to complete the process. PCR products were incubated with EZ-Vision Dye 6X (VWR) buffer. DNA bands were visualized by UV light illumination after electrophoresis on 1.2-2.5% agarose gel.

By quantitative PCR (qPCR), RNA samples isolated from different skeletal muscle samples (*m. tibialis anterior, m. pectoralis, and m. quadriceps*) were subjected to DNse treatment and the remaining mRNAs were converted to cDNA after a reverse transcription. For the qPCR assay, two different methods were used to analyse the samples in a LightCycler 480 (Roche, Basel, Switzerland) according to our existing primers. One option was to use the SYBRGreen (PCR Biosystems, Oxford, UK) mixture and the other method was the high specificity TaqMan essay (Mm00550197_m1). Light Cycler 480 SW 1.5.0 software (Roche) was used to determine the average Cp value from the cycle times of the triplicate samples. From these Cp values, the ΔCp method was used to determine the relative expression of the mRNAs of interest compared to the results of the internal control genes.

4.12 Western blot measurement

To measure protein expression, muscle samples were mechanically homogenized in lysis buffer (20 mM Tris-HCl, 5 mM EGTA, Protease Inhibitor Cocktail [Sigma, St. Louis, USA]) using a HT Mini homogenizer (OPS Diagnostics). Samples were centrifuged at 2000 g at 4°C and protein concentrations were determined from supernatants using BCA protein assay and adjusted to equal concentrations using electrophoresis sample buffer solution (20 mM Tris-HCl, pH 7.4, 10% bromphenol blue (0.01%) dissolved in SDS, 100 mM β-mercaptoethanol). The prepared samples with known concentration were boiled at 95°C for 5 min to denature the proteins. 10 μg of total protein were electrophoretically separated using a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane and non-specific binding sites were blocked using 5% skim milk powder dissolved in PBS. The membranes were incubated overnight at 4°C with the corresponding primary

antibodies. After binding, non-bound antibodies were removed by washing three times for 15 min with PBS+1% Tween-20 (PBST) and incubated with HRP-conjugated secondary antibodies for one hour at room temperature. The resulting labeling immune complexes were visualized by enhanced chemiluminescence (Thermo Fisher Scientific). Densitometric analysis of the signals was performed using ImageJ software by normalizing the detected septin7 signal to the signal strength of internal control α-actinin in the same sample. Assays were performed on at least three independent samples and the number of technical replicates was also set to three.

4.13 Statistical methods

Data are presented as mean ± standard error of the mean (SEM). Differences between control and Tamoxifen diet animals were examined by one-way ANOVA and Bonferroni post hoc multiple comparison test using GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA). Student's t-test was used to determine significance levels and $p<0.05$ was considered a significant difference.

5 Results

5.1 The striated skeletal muscle fibres express different septin isoforms

In whole lysates of human skeletal muscle samples (biopsies of *m. quadriceps femoris* obtained during amputation), almost all the septins belonging to different structural groups were detected at the mRNA level by RT-PCR reactions. Exceptions were septins 12 and 14, which could not be detected. A similar result was obtained in the analysis of skeletal muscle from newborn (4 days old) C57BL/6J mice: at the mRNA level, the presence of all septin isoforms except for 12 and 14 was detected. The expression pattern was similar in adult (4-month-old) mouse muscle, although the mRNA expression of some septin isoforms (septin5 - septin10) showed an age-dependence.

5.2 Septin7 shows variable expression during development

As the mRNA expression levels of septin showed changes during proliferation, a possible role in skeletal muscle development was suggested. We focused primarily on septin7 protein expression. Variable levels of septin7 expression

during muscle development were also observed at the protein level. The rate of septin7 protein transcription decreased progressively with age, as shown by comparing muscle samples from newborn, 4-week-old and 4-month-old mice. We observed that the expression of septin7 at a given age was independent of muscle type, as identical amounts were detected in different muscle types *(m. tibialis anterior* - TA, *m. extensor digitorum longus* - EDL and *m. soleus* - Sol). This decrease in septin7 protein expression with development is consistent with our results measured at the mRNA level. The relative expression of septin7 is remarkably high in newborn mice (NB) compared to muscle samples from later time points.

5.3 Location of septin7 in skeletal muscle fibres

To investigate the precise localization of septin7 within the skeletal muscle, enzymatically isolated individual *m. flexor digitorum brevis* (FDB) fibers from BL6 mice were subjected to immunofluorescence labeling. Since the skeletal muscle-specific α-actinin and RyR1 locations within muscle fibers are well defined, they were used as reference points in these experiments. Septin7 was visualized by immunocytochemistry and merged with α-actinin and RyR1 to determine the relative localization of septin7 with respect to the reference proteins already mentioned. The data from confocal images showed that septin7 is located along the Z-line similar to α-actinin, and we also observed septin7 labeling in the terminal cisternae characterized by RyR1.

5.4 Skeletal muscle-specific reduction of septin7 leads to an altered phenotype

To investigate the role of septin7 in skeletal muscle, a mouse model for skeletal muscle-specific knockdown of the septin7 gene was generated using the Cre/Lox system. In HSA-Cre transgenic mice, the Cre recombinase gene is driven by the skeletal muscle-specific human alpha-actin (ACTA1) promoter. These mice were mated with a mouse strain in which exon 4 encoding the GTP-binding P-loop of septin7 is flanked by a loxP sequence. Cre-mediated recombination catalyzes excision of exon 4, resulting in an additional frameshift mutation downstream of the exon. The resulting septin7 mRNAs will be truncated and no functional protein will be transcripted from them. For our *in vivo* and *in vitro* experiments, we used Cre+ hemizygous mice, which were subjected to 3 months of Tamoxifen feeding from 1 month of age prior to the studies. In these mice, Tamoxifen induced the Cre enzyme, which performed the

excision of exon 4 of septin7, thereby preventing the translation of the functional septin7 protein. Whole cell lysates from *m. quadriceps femoris* and *m. pectoralis* samples were used to measure muscle-specific downregulation of septin7 protein expression. The deletion was partial due to the multinucleated nature of the fibers, but still resulted in a significant decrease in septin7 protein expression, which was observed in each muscle type: 59 ± 8 and 63 ± 8 percent decrease compared to Cre mice in *m. quadriceps* and *m. pectoralis*, respectively. These results correlated well with the approximately 50 percent deletion determined from PCR analysis of gDNA. Cre+ mice developed a striking phenotype that manifested itself as a pronounced spinal deformity, where the spine curved outward excessively, resulting in individuals with a humped, curved-back appearance.

5.5 *In vivo* force generation is impaired in septin7 knockdown mice

In *in vivo* experiments, it was clearly observed that reduced levels of septin7 result in lower body weight and impair muscle performance. The mean grip force normalized to body weight was similar in control BL6 animals and Cre- mice, whereas significantly lower values were measured in Cre+ animals. The voluntary running tests led to similar results. All parameters of running (distance, duration, average speed, maximum speed) were significantly lower in Cre+ animals compared to BL6 animals or Cre- mice, while there was no difference in running parameters between control and Cre- animals.

5.6 *In vitro* force generation is reduced in the muscles of septin7 knockdown mice

The significant effect of the decrease in septin7 was also shown on muscle strength parameters measured by *in vitro* methods. Both individual twitch and tetanus forces were significantly reduced in EDL and Sol samples from Cre+ mice compared to the corresponding muscles of Cre- littermates. The reduction in maximal force was the result of reduced septin7 expression, as Tamoxifen treatment alone had no effect on contractile parameters. Interestingly, twitch and tetanus kinetics and fatigue were only altered in Sol of Cre+ mice. Thus, *in vivo* and *in vitro* force measurements suggest that septin7 contributes substantially to normal skeletal muscle performance. Furthermore, the expression and spatial distribution of two contractile proteins, actin and myosin (MYH4), which are important in determining muscle strength, were also examined in

individual muscle fibres isolated from FDB of Cre- and Cre+ animals. No significant changes in contractile proteins were observed; moreover, the expression pattern of the L-type calcium channel was also similar when comparing Cre- and Cre+ samples.

5.7 Reduced expression of septin7 leads to altered myofibrillar and mitochondrial structure

The structural changes induced by reduced septin7 expression in the myofibrillar system of skeletal muscle fibres were studied by electron microscopy (EM) in TA muscles. Cross-sectional EM images of Cre- animals showed myofibrils well demarcated by the sarcoplasmic reticulum (SR) located in the individual skeletal muscle fibres. However, in samples from Cre+ mice, the isolation of myofibrils was less evident. After identification of all myofibrils within an area of the actual visual field, we estimated the average total area of myofibrils within a given unit area (1 μm^2), the area and circumference of each myofibril, and the average number of myofibrils in the visual field. Both the mean area and circumference of myofibrils from Cre+ animals were significantly smaller than the corresponding parameters for myofibrils from Cre- mice. Consistent with our observations above, the number of myofibrils in a given field of view was significantly increased in samples from Cre+ mice compared to sections from Cre- animals. In the myofibrils of Cre+ mice, all the above parameters were significantly different from those of the control BL6 samples (the results of the observations in the BL6 mouse strain are only cited in this thesis but not presented here). There was no significant difference between the corresponding parameters of myofibrils isolated from BL6 animals and Cre- animals. All these results suggest that reduced septin7 expression caused a decrease in the size of individual myofibrils per area, but a significant increase in the number of myofibrils in the skeletal muscle of Cre+ mice.

The parameters mentioned above were also determined for mitochondria in cross-sectional and longitudinal images of TA muscles from different animal groups. The description of morphological changes was also performed for mitochondria. For each identified mitochondria, we calculated the area, circumference, aspect ration (AR) and form factor (FF). The calculated mean circumference and area were significantly increased in Cre+ samples compared to the data from muscles of Cre- animals and control BL6 mice samples. There was also a slight change in the relative distribution of mitochondrial area in Cre+ samples, which can be explained by the appearance of larger mitochondria. AR was significantly reduced in Cre+ samples compared to Cre- samples, whereas there was no significant difference in the calculated FF parameters. The number of mitochondria per unit area within the selected visual fields was also

significantly increased in samples from Cre+ mice compared to Cre- or BL6 mouse muscles.

Transmission electron microscopy analysis of length sections from different groups of animals revealed normal myofibrillar structure (sarcomere length, triad composition) in Cre- samples, while large mitochondrial networks were identified in most images from Cre+ muscles. The evaluation of morphological parameters in longitudinal sections revealed clear changes in mean area, perimeter, AR and FF. These parameters were significantly higher in Cre+ samples than in images of their Cre- counterparts. The shift in the relative distribution of mitochondrial area suggests that large area mitochondria appear in the muscles of Cre+ animals. The total area of mitochondria as a percentage of the total area of the visual field was also examined in BL6 and Cre- animals, but there was no significant difference in this parameter between these mice. We also determined the mitochondrial DNA content in the different muscle types *(m. pectoralis* and *m. quadriceps*). In both muscles studied, mitochondrial (16S) RNA content was strongly reduced in the muscles of Cre+ mice compared to the data from Cre- mice. These results suggest that the reduction of septin7 expression in skeletal muscle not only severely affects mitochondrial morphology but also presumably mitochondrial function.

5.8 Septin7 shows altered expression during muscle regeneration

In the TA muscles of BL6 mice, a slight sterile inflammation and consequently a mild muscle damage was induced by injection of $BaCl_2$. Cryostat sections were taken at two different time points from $BaCl_2$ injected and control muscles, which were not injected with $BaCl_2$ but injected with physiological saline. The sections were subjected to hematoxylin-eosin (HE) staining. Increased Pax7 expression due to activation of satellite cells during regeneration and centrally located nuclei in muscle fibres were observed in injected muscles compared to control samples. Levels of septin7 protein were monitored for 2 weeks during regeneration and Western blot measurements showed elevated levels at all time points examined. The elevated expression of septin7 observed following $BaCl_2$ induced muscle damage raises the possibility that cytoplasmic septins potentially contribute to regeneration through the regulation of proliferation and differentiation of newly formed muscle cells.

5.8.1 Morphological changes occur during skeletal muscle regeneration

To further elucidate the role of septin7 in regeneration, sterile skeletal muscle inflammation and thus damage was induced using the $BaCl_2$ injection method described above. Injection was performed into TA muscle of young Cre- and Cre+ mice. Sterile inflammation was induced in the left leg, while the right TA was used as a control injected with physiological saline. This type of injury is used as a simulation of muscle damage during strenuous muscle work, i.e. micro-injuries. Morphological signs of inflammation were visible in both Cre+ and Cre- mice 4 days after injection and were still present to a reduced extent 14 days after injury, as shown by HE staining. The invasion of inflammatory cells (neutrophil granulocytes, macrophages) was well observed in the $BaCl_2$ injected area, whereas no signs of inflammation were observed in control legs.

5.9 Skeletal muscle injury induces an increased expression of septin7

Confocal microscopy revealed only moderate differences in the expression pattern of septin7 filaments between Cre- and Cre+ mice. Statistical analysis of the images confirmed that protein expression in the muscle of both Cre- and Cre+ mice was increased by injection (from $67.9 \pm 5.2\%$ to $83.0 \pm 2.8\%$; and from $53.7 \pm 2.9\%$ to $80.8 \pm 5.3\%$, respectively). Significantly lower expression was detected in the muscle of Cre+ animals compared to Cre- animals ($p < 0.05$).

To further investigate the structural difference observed in immunostaining, quantitative analysis was performed at both mRNA and protein levels. No changes in the mRNA levels of septin7 were observed in Cre- mice after skeletal muscle injury during the early stages of regeneration. However, in septin7 knockdown (septin7-KD) mice, $BaCl_2$-induced injury induced an increase in septin7 mRNA on day 4 post-injection. Western blot analysis showed partially similar results. As well as at mRNA level, no difference in the expression of septin7 protein was observed in Cre- mice in response to injury. Although the mRNA level of septin7 was elevated in the injected muscle of Cre+ mice, this phenomenon was not detected in protein expression at the time point examined.

5.10 Regeneration is accompanied by an increase in the transcription factor Pax7

Dormant satellite cells (SCs) are characterized by the expression of Pax7, which shows increasing expression upon entry from dormancy to the active cell cycle. Therefore, the analysis of mRNA and protein level expression can be used to monitor regeneration processes after $BaCl_2$ injection. As expected, Pax7 mRNA levels were significantly increased 4 days after skeletal muscle injury in both Cre- and Cre+ mice. Changes in mRNA were well followed by increased expression of Pax7 protein at this time point. At the end of recovery (day 14), Pax7 mRNA was still elevated in the injected legs of Cre- and Cre+ mice. In Cre- mice, the increase in Pax7 protein was still significant. However, in the septin7 knockdown Cre+ mice, Pax7 protein no longer showed a significant increase.

5.11 Increased expression of the transcription factor myogenin in the regeneration phase

A late marker of the myogenic programme is the transcription factor myogenin, whose activation coordinates the transcription of proteins essential for differentiation. To analyse the regeneration process in more detail, we also monitored changes in miogenin. Four days after skeletal muscle injury, myogenin mRNA levels were already elevated in Cre- mice. This change was confirmed by Western blot. Partial knockdown of septin7 did not affect the changes in miogenin expression, here we also observed an increase in miogenin levels. By the end of recovery (day 14), miogenin mRNA and protein levels were down-regulated and showed no difference between control and injected legs in Cre- mice. Conversely, Cre+ animals continued to show increased expression of miogenin at the protein level, but no longer showed increased expression of miogenin mRNA levels.

5.12 Changes in laminin expression during regeneration

To monitor the progress of regeneration, mRNA and protein level of laminin was measured in each study group at 4 and 14 days after injury. Four days after skeletal muscle injury, an increase in both laminin mRNA and protein was detected in Cre- mice. However, there was no difference neither in mRNA nor in protein expression between the injured and uninjured legs of Cre+ mice. By the end of regeneration, the

increase in laminin mRNA levels in Cre- mice had subsided. Western blot analysis of the laminin protein confirmed the mRNA data. In the injured legs of Cre+ mice, laminin mRNA levels showed an increase at this time point, but laminin protein expression was delayed.

5.13 Knockdown of septin7 delays regeneration, causing morphological changes

Our data show that septin7 contributes to muscle regeneration because its partial knockdown, and thus the absence of septin7, resulted in expression and kinetic abnormalities in the molecular processes that accompany regeneration. In muscle fibers, centrally located nuclei indicate the integration of newly differentiated myoblasts from satellite cells into damaged muscle fibers. In the final step, the nuclei regain their original sarcolemmal position. To further investigate the role of septin7 in muscle regeneration, morphological analysis was also performed. The number of inflammatory cells and the number of fibres containing central nuclei were examined in HE-stained sections 14 days after injury. Fluorescent immunostaining against CD45, a common leukocyte antigen, was performed to confirm invasion by inflammatory leukocytes. Although injection of $BaCl_2$ resulted in a significant increase of inflammatory cells in both Cre+ and Cre- mice, it was significantly higher in Cre+ mice than in Cre- mice. The number of muscle fibres containing central nuclei was also significantly higher in Cre+ mice than in Cre- mice. Few centrally located nuclei were identified in the uninjected muscles.

6. Discussion

Our results suggest that septin filaments are an integral and essential part of the skeletal cytoskeleton. We already know that they can interact with other elements of the cytoskeleton, the cell membrane and can also be involved in a number of signalling pathways. Our research team has investigated the skeletal muscle and for the first time revealed the presence of different septin isoforms and the importance of septin7 for the proper functioning of the skeletal muscle, thus further expanding the cell-specific spectrum of actions of the elements of the septin system.

6.1 Expression of septin isoforms in skeletal muscle

Septins are the fourth component of the cytoskeletal system. They may be involved in the structure of skeletal muscle and may also play an important role in its function. Based on the expression patterns of septin mRNAs in mouse and human skeletal muscle samples, it can be concluded that at least one member of each homology group (SEPT2, SEPT3, SEPT6 and SEPT7) is present in striated skeletal muscle. These results suggest the presence of hexameric, higher-order oligomeric structures as confirmed by previous studies, although further structural studies are needed to understand the exact complex formation.

Septin7 showed decreasing expression with age in skeletal muscles with both fast and slow kinetics. This may suggest that its importance is more pronounced during the initial stages of muscle development. This observation is consistent with the previously recognised prominent role of septin7 in cell division. The fact that mutation and knockout of the gene for septin7, septin9 or septin11 is embryonically lethal also suggests their essential role in early development. Considering that septin7 is the only member of the SEPT7 homology group, its absence is presumed to be irreplaceable by another isoform in oligomers, because there is no other septin isoform with a similar structure. Our research group has previously shown that septin7 is essential for early skeletal muscle development and its complete absence is lethal. In this series of experiments, we have shown that even reduced expression severely impairs skeletal muscle development. Although expression of septin7 is reduced during individual development, this lower level is critical for many cellular processes. In our studies, the altered phenotype and reduced muscle strength observed in conditioned septin7 KD (Cre+) mice confirmed the theory of the importance of septin7.

The association between septins and other cytoskeletal proteins plays a prominent role in the sensing and mediation of mechanical stimuli. Septins have been shown to co-exist with actin filaments in focal adhesion complexes and stress filaments. Furthermore, septins are involved in mechanotransduction by promoting the formation of contractile actin-myosin contacts in mammalian epithelial cells and mouse cardiac myocytes.

6.2 The role of septins in mitochondrial dynamics

Our results suggest that septins play a role in the mitochondrial dynamics of mouse skeletal muscle, as area and perimeter data derived from individual mitochondria in EM images differed between control and septin7-KD induced animal samples.

Furthermore, extensive mitochondrial networks were found in the septin7 deficient fibers. However, mitochondrial DNA content was reduced despite larger mitochondria, suggesting impaired mitochondrial function in Cre+ mice. The cytoskeleton has been shown to alter mitochondrial movement and distribution in highly polar cells, and also plays a role in mitochondrial dynamics. In the absence of septin proteins, elongated mitochondria appeared in cells due to reduced mitochondrial fission, rather than defective mitochondrial fusion. It has been shown that the absence of septin2 affects mitochondrial morphology in HeLa cells. This picture is further clarified by our demonstration of a skeletal muscle-specific effect of septin7 on mitochondria. It is already known that the function of mitochondria is also mediated by the surrounding septin scaffolds. Studies have demonstrated that the co-localization of septin7 and mitochondria affects the proliferation of *Shigella flexneri* and that modification of septin expression also affects the mitochondrial morphology of *Tetrahymena thermophila*. This suggests that septins are responsible for maintaining mitochondrial stability in ciliated bacteria and protozoa. These outline an evolutionarily conserved role for septins in mitochondrial dynamics.

6.3 The role of septin7 in skeletal muscle regeneration

In our experiments, we found that the expression of septin7 is increased by mild muscle damage induced by $BaCl_2$ injection. We monitored the occurrence and extent of injection-induced inflammation and subsequent regeneration by detecting the presence of Pax7-positive satellite cells and found a clear correlation in the time course of the expression pattern of the two molecules. This observation was in agreement with previous findings on Pax7. On average, myogenic regeneration is most intense 4 days after injury, so that the increase in expression of both Pax7 and septin7 was greatest during this phase. By 14 days after necrosis, regenerative processes were essentially complete and the expression of both molecules had returned to baseline levels. Since the appearance of the transcription factor Pax7 and the centrally located nuclei in the fiber are accepted markers of muscle regeneration, we conclude that the increased expression of septin7 may also be related to the progression of muscle regeneration. Although these results demonstrated the importance of septin filaments in the regeneration steps following muscle injury, further experiments were needed to clarify their exact role.

To investigate the role of septin7 in muscle regeneration, we used our conditioned KD mouse strain. A 40% decrease in the level of septin7, in addition to the

already mentioned spinal deformity and muscle strength loss, impaired the ability to regenerate, which significantly affected the dynamics of regeneration.

The differences seen on confocal images, i.e. the increase in the expression of septin7 following inflammation, were also reflected in changes in protein and mRNA levels. Both animal strains (Cre- and Cre+) showed an increase in the amount of septin7 mRNA after injection, but these changes were more pronounced in Cre+ mice. At the mRNA level, this increase was significant in Cre+ animals as early as day 4, whereas at the protein level it was only detectable at day 14. As expected, mRNA levels in the control leg of the Cre+ animal were significantly lower in absolute terms than in the Cre- animal. After the onset of regeneration, there was a rapid increase in the level of septin7 mRNA in Cre+ mice, which was thought to provide the increased amount of septin7 required for regeneration, but the increase in protein levels occurred only after a few days delay. In this process, inflammation may have caused an overcompensated production of septin7, which was also trying to compensate for the previous deficiency. It is assumed that such a large increase in the mRNA of septin7 is necessary, which underlines its role in the process. It is also likely that a given amount of septin7 is essential for the regeneration process, and if the fibres are slow to produce it, the dynamics of the process will be shifted.

Pax7 as a major satellite cell activation marker is essential for the initiation of the regeneration phase. Interestingly, for Pax7, there was no significant difference in protein or mRNA levels between the two groups. This may suggest that the regenerative capacity of Cre- and Cre+ mice is initially similar, as Pax7 expressed by satellite cells and required for muscle regeneration reached almost identical levels. Furthermore, the fact that the Cre recombinase gene was driven by the skeletal muscle-specific alpha-actin promoter, which is only active in differentiated fibres, may also contribute to this. Consequently, the expression of septin7 was reduced only in skeletal muscle fibres and did not affect satellite cells, as Pax7-positive satellite cells express a different type of actin. In all cases, injury induced an increase in Pax7 levels, indicating the beginning of the regeneration cascade.

We also examined the levels of another transcription factor, myogenin, in Cre- and Cre+ animals. Myogenin is involved in the terminal differentiation of immature muscle cells, directing the transcription of proteins required for this process. In the non-injected samples, we did not see any differences between Cre+ and Cre- animals at neither mRNA nor at protein level. However, the increased myogenin protein expression induced by $BaCl_2$ injection persisted in Cre+ animals even at day 14, causing a change in the dynamics of regeneration and suggesting a prolonged muscle fibre regeneration. In Cre- mice, the previous high mRNA expression was no longer clearly detectable at day 14. This may suggest that septin7 may play a role in later steps of the

process. Signal transduction processes are initiated but not switched off after the physiological time period due to the slowed down process.

This is not the only indication that knockdown of septin7 has a negative effect on the dynamics of muscle healing.

Laminin is an ECM molecule that was significantly upregulated in Cre- mice 4 days after injury, whereas partial knockdown of septin7 in Cre+ individuals prevented the timely expression of laminin. In Cre+ mice, the increase in laminin mRNA only started in the 14-day-old samples. The increase in protein amount presumably occurred after day 14, a significant delay compared to Cre- animals. This shift in laminin expression is due to the altered cytoskeleton formed in the absence of septin7. Without a stable, mature scaffold, ECM synthesis is not justified, since hemidesmosomes that have not yet formed or are not functional in the absence of septin cannot bind to ECM elements such as laminin.

Based on our experiments, we believe that reduced septin7 expression significantly prolongs the regeneration phase. Tamoxifen alone, used as an inducer of the Cre-lox system in our experiments, slowed down the regeneration process to a small extent, due to its estrogen analogue nature and effect. Therefore, in our experiments, the comparison was always made between Cre- and Cre+ individuals. There was a high diversity in the response to induced inflammation between individuals and therefore the regeneration stages may have been slowed down to varying degrees.

To summarise our experience, an increase in the amount of septin7 was observed in all strains, although to a variable extent. This suggests that protein is essential for regeneration. However, we can also conclude that although the transcription of septin7 is slower in Cre+ animals, it reaches the expression levels of Cre- animals over a longer period of time, thus supporting the hypothesis that a given amount of septin7 is essential for muscle recovery. These data suggest that terminal differentiation of muscle fibres does not occur in the absence of septin7. This is also reflected by the prolonged elevated myogenin levels, which may be due to the fact that the desired degree of differentiation cannot be achieved at low levels of septin7. The delayed expression of laminin protein in Cre+ animals is also consistent with this picture, confirming the importance of septin7 and its effect on the subsequent steps of regeneration.

In addition to expression studies, morphological changes accompanying regeneration were also analysed. The number of inflammatory cells increased in all samples as a consequence of the injury, due to the sterile inflammation. Under control conditions, the number of white blood cells decreased after the inflammation. It should be noted here that the detected immune cells, especially macrophages, play an important role not only in the removal of damaged tissue but also in the induction of

regeneration. In Cre+ individuals, the leukocyte infiltration rate was significantly higher even at day 14, indicating that the lack of septin7 delayed healing.

In addition to immune cell infiltration, the number of nuclei centrally located in the fibre supported our hypotheses. Such localization of nuclei is a typical indicator of muscle regeneration, because they indicate the high amount of protein synthesis required for healing and differentiation. The number of muscle fibers containing central nuclei is also significantly higher in Cre+ mice than in Cre- mice, further emphasizing the elongation of regeneration kinetics and the role of septin7 in muscle healing.

7. Summary

Studies with the cytoskeletal protein septin7 in genetically modified mice have shown a reduction in muscle strength and a spinal deformity, suggesting a significant role for this protein in skeletal muscle. This effect is dual in nature. Due to structural defect loss of function could be observed in the septin system, and mitochondrial function is impaired as well. The structural modification of individual mitochondria and the formation of interconnected mitochondrial networks may suggest that the septin system and the process of mitochondrial fission and division are closely related. These morphological changes may have contributed to further rearrangements of the fibre structure and to the impairment of mitochondrial function. The expression of septin7 showed an age-dependence, with elevated expression of septin7 in young individuals, which could be attributed to its important role in cell division. With age, its expression decreases and its function is more focused in membrane organization, differentiation, cell-cell contacts and internal structure formation. Each of these processes plays a major role in the regeneration process, and as a result, the lack of septin7 can interfere with muscle recovery at several points. In our experiments, we demonstrated that the lack of septin7 resulted in altered expression of markers of muscle healing, which may indicate that the phases of regeneration are slowed down and recovery is delayed.

It should be noted that the effect of septin7 is not necessarily direct in these processes. Several studies have also highlighted the role of the actin scaffold and the importance of microtubule dynamics in muscle fibre formation. Furthermore, it has been shown that the interactions of cytoskeleton members have a significant influence on each other. Moreover, not only cytoskeleton members but also other signalling proteins may form complexes with the septin7 protein.

A more precise understanding of the role of septin7 and the exploration of its interaction partners may help to more fully map the process of muscle regeneration and

muscle force generation. This knowledge could contribute to the development of therapies for muscular atrophy and age-related muscle weakness, as well as to the prevention of muscle atrophy in microgravity, thus improving the quality of life of the patients concerned.

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List of publications related to the dissertation

1. **Szabó, L.**, Telek, A., Fodor, J., Dobrosi, N., Dócs, K., Hegyi, Z., Gönczi, M., Csernoch, L., Dienes, B.: Reduced Expression of Septin7 Hinders Skeletal Muscle Regeneration.
   Int. J. Mol. Sci. 24 (17), 13536, 2023.
   DOI: http://dx.doi.org/10.3390/ijms241713536
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2. Gönczi, M., Ráduly, Z., **Szabó, L.**, Fodor, J., Telek, A., Dobrosi, N., Balogh, N., Szentesi, P., Kis, G., Antal, M., Trencsényi, G., Dienes, B., Csernoch, L.: Septin7 is indispensable for proper skeletal muscle architecture and function.
   eLife. 11, e75863, 2022.
   DOI: http://dx.doi.org/10.7554/eLife.75863
   IF: 7.7

List of other publications

3. Ráduly, Z., **Szabó, L.**, Dienes, B., Szentesi, P., Bana, Á. V., Hajdú, T., Kókai, E., Hegedűs, C., Csernoch, L., Gönczi, M.: Migration of Myogenic Cells Is Highly Influenced by Cytoskeletal Septin7.
   Cells. 12 (14), 1825, 2023.
   DOI: http://dx.doi.org/10.3390/cells12141825
   IF: 6 (2022)

4. Dienes, B., Bazsó, T., **Szabó, L.**, Csernoch, L.: The Role of the Piezo1 Mechanosensitive Channel in the Musculoskeletal System.
   Int. J. Mol. Sci. 24 (7), 6513, 2023.
   DOI: http://dx.doi.org/10.3390/ijms24076513
   IF: 5.6 (2022)

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5. Gönczi, M., Csemer, A., **Szabó, L.**, Sztretye, M., Fodor, J., Pocsai, K., Szenthe, K., Keller-Pintér, A., Köhler, Z. M., Nánási, P. P., Szentandrássy, N., Pál, B., Csernoch, L.: Astaxanthin Exerts Anabolic Effects via Pleiotropic Modulation of the Excitable Tissue.
Int. J. Mol. Sci. 23 (2), 917, 2022.
DOI: http://dx.doi.org/10.3390/ijms23020917
IF: 5.6

6. Singlár, Z., Ganbat, N., Szentesi, P., Osgonsandag, N., **Szabó, L.**, Telek, A., Fodor, J., Dienes, B., Gönczi, M., Csernoch, L., Sztretye, M.: Genetic Manipulation of CB1 Cannabinoid Receptors Reveals a Role in Maintaining Proper Skeletal Muscle Morphology and Function in Mice.
Int. J. Mol. Sci. 23 (24), 1-21, 2022.
DOI: http://dx.doi.org/10.3390/ijms232415653
IF: 5.6

7. **Szabó, L.**, Balogh, N., Tóth, A., Angyal, Á., Gönczi, M., Csiki, D. M., Tóth, C., Balatoni, I., Jeney, V., Csernoch, L., Dienes, B.: The mechanosensitive Piezo1 channels contribute to the arterial medial calcification.
Front. Physiol. 13, 1037230, 2022.
DOI: http://dx.doi.org/10.3389/fphys.2022.1037230
IF: 4

8. Dienes, C., Hézső, T., Kiss, D. Z., Baranyai, D., Kovács, Z. M., **Szabó, L.**, Magyar, J., Bányász, T., Nánási, P. P., Horváth, B., Gönczi, M., Szentandrássy, N.: Electrophysiological Effects of the Transient Receptor Potential Melastatin 4 Channel Inhibitor (4-Chloro-2-(2-chlorophenoxy)acetamido) Benzoic Acid (CBA) in Canine Left Ventricular Cardiomyocytes.
Int. J. Mol. Sci. 22 (17), 9499, 2021.
DOI: http://dx.doi.org/10.3390/ijms22179499
IF: 6.208

9. Sztretye, M., **Szabó, L.**, Dobrosi, N., Fodor, J., Szentesi, P., Almássy, J., Magyar, Z. É., Dienes, B., Csernoch, L.: From Mice to Humans: an Overview of the Potentials and Limitations of Current Transgenic Mouse Models of Major Muscular Dystrophies and Congenital Myopathies.
Int. J. Mol. Sci. 21 (23), 8935, 2020.
DOI: http://dx.doi.org/10.3390/ijms21238935
IF: 5.924

10. Sztretye, M., Singlár, Z., **Szabó, L.**, Angyal, Á., Balogh, N., Vakilzadeh, F., Szentesi, P., Dienes, B., Csernoch, L.: Improved Tetanic Force and Mitochondrial Calcium Homeostasis by Astaxanthin Treatment in Mouse Skeletal Muscle.
Antioxidants. 9 (2), 98, 2020.
IF: 6.312

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11. Ráduly, Z., **Szabó, L.**, Madar, A., Pócsi, I., Csernoch, L.: Toxicological and Medical Aspects of Aspergillus-Derived Mycotoxins Entering the Feed and Food Chain.
Front. Microbiol. 10, 2908, 2020.
DOI: http://dx.doi.org/10.3389/fmicb.2019.02908
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