Short thesis for the degree of doctor of philosophy (PhD)

Investigation of the role of the main regulatory proteins in the Ca^{2+} homeostasis of chondrogenic progenitor cells and skeletal muscle cells

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Introduction

The global prevalence of musculoskeletal disorders is constantly rising owing to unfavourable changes in the population of developed countries. Osteoarthritis (OA) is the most common form of chronic musculoskeletal disorders. At the clinical stage, inflammation of the synovial membrane is often present, which further influences chondrocyte metabolism to enhance catabolism and reduce anabolism, resulting in altered extracellular matrix (ECM) homeostasis and composition. Indeed, OA-affected cartilage matrix has long been known to undergo profound changes in terms of collagen, glucoseaminoglycan and water content, which in turn alters the osmolarity of the matrix and its unique ionic milieu, and affects chondrocyte metabolism. We have recently documented a distinct cell population with migratory potential in OA-affected articular cartilage. These chondrogenic progenitor cells (CPCs) exhibit certain stem cell surface markers and posses stem cell-like characteristics such as clonogenicity, multipotency and migratory activity. CPCs express intermediate levels of both the osteogenic and chondrogenic transcription factors Runx2 and Sox9, respectively, indicating that they are likely derived from the osteochondroprogenitor lineage. Although CPCs *in vivo* have only limited regeneration capacity, a better understanding of their biological characteristics and targeted manipulation of certain pathways may lead to enhanced synthesis and regeneration of cartilage ECM. Since arthritic cartilage matrix undergoes profound changes especially at the late stages of the disease, it is logical to assume that cells in OA-affected cartilage may be characterised by a unique assembly of plasma membrane ion channels and transporters that regulate their function and phenotype.

Skeletal muscle development is based on the fusion of myoblasts into a myotube. This multinucleated syntitium contains a complex and sophisticated internal membrane system called sarcoplasmic reticulum (SR) considered as a

specialized form of endoplasmic reticulum. The SR is an attribute of muscle entity and predominantly regulates calcium movements during contraction-relaxation cycle; Ca^{2+} is released from the SR into the sarcoplasmic space where it triggers muscle contraction then it is reuptaken during the relaxation period and stored in the SR. There are proteins in the SR specialized for this activity; the main players being the ryanodine receptor (RyR) through which Ca^{2+} is released into the sarcoplasm, the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that reuptakes Ca^{2+} into the SR from the sarcoplasm, and calsequestrin (CSQ) that binds stored Ca^{2+} in the SR lumen. The three main SR proteins are expressed in developmental isoforms in fetal/postnatal stages and in myotubes of mammals (RyR, CSQ, SERCA1b). The ratio and the functional differences of these proteins compared to the adult isoforms are not entirely known although it could probably be important for better understanding the mechanism of muscle differentiation and store-operated calcium entry (SOCE). SERCAs are the main Ca^{2+} pumps which decrease the intracellular Ca^{2+} level by reaccumulating Ca^{2+} into the sarcoplasmic reticulum. The neonatal SERCA1b is the major Ca^{2+} pump in myotubes and young muscle fibers. To understand its role during skeletal muscle differentiation its synthesis has been interfered with specific shRNA sequence. Stably transfected clones showing significantly decreased SERCA1b expression (cloneC1) were selected for experiments.

Our aims

Based on the above findings it can be hypothesised that alterations of the molecular “calcium toolkit” that regulates Ca^{2+} homeostasis are also present in inflammatory chondrocytes and other cell types in OA-affected cartilage. These alterations can serve as a background for modified metabolism and altered physiological properties of these cells. In particular, ATP is also known to be an important mediator in various inflammatory conditions; in particular, ATP levels can significantly increase following its release by damaged cells. In turn,

elevated levels of ATP provoke the activation of purinergic receptors, mainly P2X and P2Y receptors. In particular, synovial fibroblasts obtained from OA patients were reported to express $P2X_1$ and $P2X_3$ receptors at high density that modulated some functional responses closely associated with inflammation. We aimed to provide a detailed understanding of the Ca^{2+} homeostasis in CPCs. We characterised calcium influx functions through the plasma membrane with special emphasis on purinergic signalling; furthermore, we also mapped Ca^{2+} release pathways from internal Ca^{2+} stores, as well as Ca^{2+} elimination functions. Moreover, the contribution of these pathways to the regulation of low-frequency spontaneous Ca^{2+} oscillations is also discussed.

The further aim was to explore the function of SERCA1b, a major calcium pump of *in vitro* myotubes and embryonic/postnatal human and rodent muscles. SERCA1b mRNA is spliced from the transcript of the SERCA1 gene by skipping exon 22 while in the adult SERCA1a mRNA each exon remains . Since the first stop codon is in exon 22, the translation of SERCA1b terminates in exon 23 using the second stop codon. As a result, the SERCA1b protein has an eight amino acid long tail instead of the C-terminal glycine of the SERCA1a protein. SERCA1a is expressed in adult fast type skeletal muscle, however, no functional difference could be observed in the Ca^{2+} transport and affinity if compared to SERCA1b when their corresponding cDNAs are expressed in COS-1 cells. SERCA1 knock-out mice (expressing neither SERCA1a nor SERCA1b) die in respiratory failure and cyanosis shortly after birth probably because of insufficient function and development of the diaphragm, which has been shown to express SERCA1b as the main SERCA1 isoform in neonatal mice. Interestingly, the expression of SERCA1b is under strict posttranscriptional control; although its mRNA is upregulated in stretch and denervation of adult muscle, the protein is expressed only in developing or regenerating muscle independently of whether it is becoming a slow or fast type.

Materials and methods

Cell cultures

CPCs were isolated, immortalized and cultured as described by Koelling et al. In these experiments, the CPC531 clone was used. CPCs were routinely maintained in DMEM containing 1 $g \cdot L^{-1}$ glucose and 10% FBS until approx. 80% confluence and were passaged. For calcium measurements, cells were harvested and transferred onto 30-mm round cover slips placed into 35-mm plastic Petri dishes at a density of 10^3 cells $\cdot$ cm^{-2}. Cultures were used only until they reached approx. 80% confluence. The medium was changed on every second day.

C2C12 mouse skeletal muscle cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum(FBS), 50 U/ml penicillin, and 50 g/ml streptomycin and were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 and 80% humidity in a humidified incubator. Differentiation of non-transfected, and transfected cells was induced at 80% confluency by exchanging the culture medium for DMEM supplemented with 5% horse serum (HS), 2% FBS and penicillin–streptomycin.

Transfection

RNA interference was applied in order to reduce endogenous SERCA1b expression. The sequence targeted for SERCA1b silencing was 5-*ctatctggaggatccagaa*-3, corresponding to the last 11 bases of exon 21 and the first 8 bases of exon 23. These fragments are contiguous only in the spliced SERCA1b mRNA after skipping exon 22. The chosen shRNA cassette sequence besides the sense (5'*ctatctggaggatccagaa*) and antisense (5'*ttctggatcctccagatag*) region contains a loop and termination sequence resulting in a hairpin siRNA. Blast filtering ensured that this sequence has homology only with SERCA1b and not with any other known gene. Scrambled shRNA was used to demonstrate that

the target specific shRNA did not induce a nonspecific effect on gene expression. These shRNA sequences were cloned into pLKO.1-puro-CMV-tGFP expression vector. Stable transfection was performed in Opti-MEM reduced serum content medium using Lipofectamine 2000 reagent for 2.5 h at 37°C. Cells were allowed to express the introduced sequence for 48 h in growth medium then were selected in DMEM containing 1.5 μg/ml puromycin. After 14–15 days, single colonies were isolated and experiments were carried out on separated clones of SERCA1b transfected cells. Pool of scrambled shRNA transfected and non-transfected parental cells were used as control.

Immunostaining

Cultured cells were washed with ice-cold phosphate-buffered saline (PBS), fixed with −20°C 100% methanol for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 1% bovine serum albumin (BSA) diluted in PBS (blocking solution) for 30 min at room temperature. Cells were then incubated for 4 hours at 4°C with the anti-desmin primary antibody (dilution was 1:500 in blocking solution). Then fluorescein (FITC) labeled anti-mouse secondary antibody was applied for 1 h at room temperature. Vectashield mounting medium with DAPI was used for visualization of nuclei. Images were taken using LSM 510 META confocal microscope.

SDS–PAGE and Western blot analysis

Total cell lysates were examined by Western-blot analysis. The CPC cultures were washed with physiological NaCl solution and were harvested. After centrifugation at 2000 ×*g* for 10 min at room temperature, cell pellets were suspended in 100 μL of RIPA homogenization buffer. Samples were stored at –70 °C. Suspensions were sonicated by pulsing burst for three times 30 s by 50 cycles using an ultrasonic homogenizer. Samples for SDS–PAGE were prepared by adding 1/5 volume of 5× concentrated Laemmli's electrophoresis sample

buffer to cell lysates and heated at 95 °C for 5 min. 50 μg of protein was separated by 7.5% SDS–PAGE gel for immunological detection of key proteins involved in Ca^{2+}-homeostasis of CPC cells. Proteins were transferred to nitrocellulose membranes by using a Bio-Rad Trans-Blot Turbo system. After blocking in 5% non-fat dry milk in PBS, membranes were incubated with primary antibodies overnight at 4 °C. After washing for 30 minutes in PBST, membranes were incubated with the HRP-conjugated secondary antibodies. Membranes were developed by enhanced chemiluminescence reaction according to the instructions of the manufacturer.

Total cell lysates of C_2C_{12} myotubes for SDS–PAGE were prepared by the addition of 1/5 volume of 5-fold concentrated electrophoresis sample buffer to cell lysates and boiled for 5 min at 80 °C. 30 μg of protein was separated by 7.5% SDS–PAGE gel for immunological detection of examined proteins. Samples were transferred electrophoretically to nitrocellulose membranes. After blocking with 5% non-fat dry milk in PBS, membranes were incubated with the appropriate primary antibodies overnight at 4 °C. After washing three times for 10 min with PBST (PBS supplemented with 0.1% Tween 20), membranes were incubated with a secondary antibody. Signals were detected by enhanced chemiluminescence (ECL) reaction. The intensity of the specific bands and the background from the same image were measured by ImageJ. The background values were then substracted. The values were normalized to those obtained for actins of the same samples.

mRNA expression analysis using reverse transcription followed by PCR (RT-PCR)

Subconfluent colonies of CPC cells cultured in 150 cm^2 plastic cell culture dishes were washed three times with RNase-free physiological NaCl, dissolved in TRIzol, and following addition of 20% RNase-free chloroform samples were centrifuged at 10,000 ×*g* for 15 min at 4 °C. Total RNA-

containing samples were incubated in 500 µL RNase-free isopropanol at –20 °C for 1 h, total RNA was dissolved in nuclease-free water and stored at –70 °C. The composition of the assay mixture (20 µL) for reverse transcriptase (RT) reactions was as follows: 1000 ng total RNA; 0.25 µL RNase inhibitor; 2 µL random primers; 0.8 µL dNTP Mix (4 mM); 50 units (1 µL) of MultiScribe™ RT in 1× RT buffer. cDNA was transcribed at 37 °C for 2 hours.

Amplifications of specific cDNA sequences were carried out using specific primer pairs that were designed by Primer Premier 5.0 software PCR reactions were carried out in a final volume of 25 µL containing 1–1 µL forward and reverse primers (10 µM), 0.5 µL cDNA, 0.5 µL dNTP Mix (200 µM), and 1 unit (0.2 µL) of GoTaq® DNA polymerase in 1× Green GoTaq® Reaction Buffer in a programmable thermal cycler with the following protocol: 2 min at 95 °C for initial denaturation followed by 35 repeated cycles of denaturation at 94 °C for 30 s, primer annealing for 45 s at an optimised temperature for each primer pair, and extension at 72 °C for 90 s. After the final cycle, further extension was allowed to proceed for another 7 min at 72 °C. PCR products were analysed using horizontal gel electrophoresis in 1.2% agarose gels containing ethidium bromide at 100 V constant voltage.

For RT-PCR analysis, C_2C_{12} colonies were washed three times with ice cold PBS, snap-frozen in liquid nitrogen and stored at −70°C. Total RNA was isolated from myotubes. Assay mixture (20 µl) for reverse transcriptase reaction contained 500 ng RNA, 0.25 µl RNase inhibitor, 0.25 µl oligo (dT), 2 µl dNTP (200 µM), 1 µl M-MLV RT in 1 × RT buffer. Amplifications of specific cDNA sequences were carried out using specific primer pairs that were designed by Primer Premier 5.0 software. PCR reactions were allowed to proceed in a final volume of 50 µl (containing 2 µl forward and reverse primers, 1 µl dNTP (200 µM), and 0.5 µl Promega GoTaq® DNA polymerase (in 1 × reaction buffer) with the following settings: 2 min at 95°C for initial denaturation followed by

repeated cycles of denaturation at 94°C for 1 min, primer annealing for 60 s at an optimized temperature for each primer pair and extension at 72°C for 1 min 30 s. After the final cycle, further extension was allowed to proceed for another 10 min at 72°C. PCR products were analyzed using 1.5% agarose gel at 100 V constant voltage.

Single cell fluorescent Ca^{2+} measurements

Measurements were performed using the calcium-dependent fluorescent dye Fura-2. Cultures were transferred to 1 mL fresh DMEM containing 5 μL Fura-2-acetoxy-methylester and 3 μL neostigmin and incubated in a CO_2 incubator at 37 °C for 1 h. Fura-2-loaded cells were then placed on the stage of an inverted fluorescent microscope and viewed using a 40× oil immersion objective. Calcium imaging was performed in normal Tyrode's (containing 137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11.8 mM HEPES; 1 g · L^{-1} glucose; pH 7.4) or in Ca^{2+}-free Tyrode's solution (containing 5 mM EGTA, without $CaCl_2$). Excitation wavelength was altered between 340 and 380 nm (F_{340} and F_{380}) by a microcomputer-controlled dual-wavelength monochromator equipment (DeltaScan; Photon Technologies International, New Brunswick, NJ, USA). Emission was detected at 510 nm at 10 Hz acquisition rate using a photomultiplier. Background fluorescence was subtracted on-line from F_{340} and F_{380} signals by the data acquisition software. Intracellular [Ca^{2+}] was calculated from the ratio of measured fluorescence intensities ($R=F_{340}/F_{380}$) as described by Grynkiewicz and colleagues. The measuring bath was constantly perfused with normal Tyrode's solution at a rate of 2 mL · min^{-1}. Test solutions were directly applied to the cells through a perfusion capillary tube with an internal diameter of 250 μm at a rate of 1.5 μL · s^{-1}, using a local perfusion system. All measurements were performed at room temperature.

Examination of cell proliferation and differentiation

Cultures of C2C12 cells were photographed daily from the 1st to the 5th day after seeding using a Canon EOS-300D digital single lens reflex camera mounted on a phase contrast microscope. Culturing was performed in parallel in the presence of 200 nM cyclosporineA (CSA), solution was changed every 2nd day. Photos of five fields of view per culture were taken every day. Myogenic nuclei were marked manually and morphometric analysis was done on these images. To detect the quantitative parameters of differentiated myotubes 15-15 random fields of view were examined from 3 independent culturing. Cultures on the 5th day of differentiation were fixed, and the number of DAPI-stained nuclei were counted manually, the diameter was measured by Image Browser.

Calcineurin activity assay

For *in vitro* CaN activity assays, cells were washed in physiological NaCl solution and were harvested. After centrifugation, cell pellets were suspended in 100 μL of homogenization RIPA buffer containing protease inhibitors. Samples were stored at –70°C. Suspensions were sonicated using pulsing bursts for 30 s at 40 A. After centrifugation at 10,000×*g* for 10 min at 4°C, supernatants with equal protein concentrations were used for enzyme activity measurements.

Results I.

Spontaneous Ca^{2+} transients in CPCs are mediated by internal stores via IP_3R

The dynamics of intracellular Ca^{2+} levels in CPCs was analysed in Fura-2 loaded cells during 1000-sec-long intervals. In the majority of cells observed spontaneous Ca^{2+} transients. The average frequency of transients was $1.3\times10^{-3}\pm1.3\times10^{-4}$ Hz; the amount of Ca^{2+} liberated during each transient was 23.6±5.4 μM · s; the average amplitude of Ca^{2+} spikes was 116.6±11.6 nM and the rate of rise of transients was 2.18±0.5 nM · s^{-1} (in each case, *n*=20). To establish whether the primary source of elevated cytosolic Ca^{2+} levels was an influx through plasma membrane Ca^{2+} channels or a release from internal Ca^{2+} stores, further experiments were performed. When CPCs were bathed in Ca^{2+}-free Tyrode's during single cell Ca^{2+} measurements, spontaneous Ca^{2+} transients were still detectable (in 9 of 10 cells investigated; 90%), although with gradually decreasing amplitudes and frequencies. The average frequency and amplitude of these spikes were $1.4\times10^{-3}\pm1.5\times10^{-4}$ Hz and 78.5±9.8 nM, respectively, and 14.7±2.8 μM · s Ca^{2+} was found to be liberated during each Ca^{2+} transient. The rate of rise of transients was 1.67±0.4 nM · s^{-1} (in each case, *n*=9). While significant differences were found between the amplitudes of transients in Ca^{2+}-free medium compared to controls (*P*=0.02), neither the amount of liberated Ca^{2+} nor the rate of rise of the transients were statistically different (*P*=0.26 and *P*=0.49, respectively). These results suggested that although a Ca^{2+} influx through plasma membrane ion channels contributed to the amplitude of Ca^{2+} spikes, the primary source of Ca^{2+} to generate spontaneous Ca^{2+} transients could probably be the internal stores.

Further experiments were carried out. When the SERCA inhibitor CPA (10 μM) was added to the bath solution with 1.8 mM Ca^{2+} and concurrent inhibition of store-operated Ca^{2+} entry (SOCE) by $LaCl_3$ (500 μM) and YM-

58483 (1 μM) was applied, spontaneous activity was completely abolished (*n*=10). This observation suggested that even though Ca^{2+} was available extracellularly, the fact that internal stores were depleted and store replenishment was concurrently blocked was sufficient to completely abrogate spontaneous transients, which supports the idea that the function of internal Ca^{2+} stores was of key importance in this process. To test the role of IP_3 receptors, the aspecific IP_3R blocker 2-APB (75 μM) was administered to the bath solution during single cell Ca^{2+} measurements with no external Ca^{2+} available. In this case, no spontaneous activity could be detected (*n*=10), which implicates that Ca^{2+} release through IP_3R subtype(s) was required for the induction of transient rise in cytosolic Ca^{2+} . In contrast, application of 30 mM caffeine had no effect on spontaneous Ca^{2+} events.

Functional characterisation of internal Ca^{2+} stores

IP_3R in the ER membrane seemed to play a determining role in generating Ca^{2+} transients, we undertook to characterise the internal Ca^{2+} stores. First, by using specific primer pairs, we performed an mRNA transcript analysis of key molecules and found that although all three IP_3R isoforms were observed in CPCs, no specific bands for RyR subtypes could be detected.We also screened molecules that orchestrate SOCE and found that both isoforms of the ER Ca^{2+} sensor molecule STIM (STIM1 and STIM2), and all three isoforms of the plasma membrane CRAC channel Orai (Orai1, Orai2 and Orai3) mRNAs were expressed by CPCs.

The next step was to identify their protein level expression. By applying isotype-specific IP_3R antisera, all IP_3R subtypes (IP_3R1, IP_3R2 and IP_3R3) were found to be expressed by CPCs as revealed by Western blot analyses. Of the molecules that orchestrate SOCE, only STIM1 and Orai1 were detectable at the protein level.

The functionality of these proteins was investigated during single cell fluorescent Ca^{2+} recordings. When the SERCA blocker CPA (10 μM) was

administered to cells in Ca^{2+} free Tyrode's, an approx. 60 nM rise in resting Ca^{2+} levels was observed, caused by Ca^{2+} release from the internal stores probably *via* IP_3Rs and concurrent inhibition of Ca^{2+} re-uptake, followed by a stable decline owing to the activity of Ca^{2+} elimination pathways other than SERCA. From these data, the releasable Ca^{2+} content of internal Ca^{2+} stores could be calculated, which was 22.2±2.4 μM · s (*n*=25). When the bath solution was replaced by 1.8 mM Ca^{2+} containing Tyrode's, a large (approx. 150 nM) transient Ca^{2+} influx was detected, demonstrating store-operated Ca^{2+} entry. When the experiment was repeated with concurrent application of non-specific SOCE blockers YM-58483 (1 μM) and $LaCl_3$ (500 μM), a significantly smaller amplitude ($\Delta[Ca^{2+}]_i$: 131±16.4 nM *vs*. 69.2±10.6 nM; *n*=13 and 8; *P*=0.005) was recorded after Ca^{2+} became available in the bath solution; however, the differences in rate of rise (2.04±0.5 nM · s^{-1} *vs*. 0.93±0.2 nM · s^{-1}; *n*=13 and 8; *P*=0.16) were not statistically significant. This is probably a consequence of large individual variations among cells.

Extracellular administration of nucleotides evoke Ca^{2+} transients in CPCs

We aimed to identify signalling pathways that couple extracellular signals to the IP_3R. According to the results of Kawano and colleagues, periodic Ca^{2+} transients in human mesenchymal stem cells (MSCs) could be attributed to an autocrine/paracrine purinergic signalling loop. Following this framework of reasoning, we also hypothesised a similar purinergic autostimulation in migratory chondroprogenitor cells. We first carried out a comprehensive mRNA transcript screening for all purinergic receptor mRNAs involved in Ca^{2+} homeostasis and found that of the A1 adenosine receptor subtypes A_1, A_{2A} and A_{2B}, but not A_3 adenosine receptor transcripts; of the P2X ionotropic purinergic receptor subtypes $P2X_1$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$, but not $P2X_2$ and $P2X_3$; and mRNAs for all G_q-copuled P2Y metabotropic purinergic receptor subtypes were detectable using RT-PCR .

To assess the functionality of these receptors, P1 and P2 receptor agonists

were administered to CPCs during single cell calcium measurements. When the effect of ATP (180 μM), a general P2 receptor agonist was tested, rapid and large amplitude (338.1±28.5 nM; *n*=44), repetitive Ca^{2+} transients could be detected in normal (1.8 mM [Ca^{2+}]) Tyrode's solution (*n*=44 of 48 cells; 91%), reflecting on the activity of P2 purinergic receptors. When ATP was administered in 0 mM external Ca^{2+}, repetitive transients could still be evoked (*n*=35 of 36 cells; 97%), but with significantly lower amplitudes compared to the control (204.6±25 nM; *n*=35; *P*=0.0009). Similarly, there was statistically significant difference between the rate of rise of transients in control and Ca^{2+}-free solutions (72.9±12.6 nM · s^{-1} *vs*. 35.4±8.8 nM · s^{-1}; *P*=0.02). The above data suggested the contribution of both ionotropic P2X and metabotropic P2Y purinergic receptors and/or SOCE. However, when the IP_3R inhibitor 2-APB was co-applied with ATP, transients could be evoked only in a few cases (*n*=2 of 19 cells.

The effects of various P2Y receptor agonists were tested during single cell Ca^{2+} measurements. While UTP (100 μM) and ADP (100 μM) evoked prominent Ca^{2+} transients (324.5±20.5 nM and 284±25 nM, respectively) in most of the cells examined (*n*=13 of 15 and 18 of 20 cells, respectively), no response was detected when UDP (100 μM) was administered to cells (*n*=0 of 11 cells). Furthermore, the functionality of P1 adenosine receptors was also confirmed as local application of adenosine (100 μM) evoked Ca^{2+} transients (average amplitude: 258.4±26.7 nM) in most of the cells investigated (*n*=16 of 18 cells), while co-application of adenosine with the P1 receptor blocker caffeine (30 mM) eliminated this response (*n*=0 of 10 cells).

Functional expression of P2 purinergic receptors

mRNAs of various P2 purinergic receptor subunits were detected in the transcriptome of CPCs. Next, we applied immunoblot analyses to confirm the presence of these molecules at the protein level. Of the P2Y receptors, only the G_q protein-coupled P2Y receptor subtypes (*i.e.* $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and

$P2Y_{11}$) were investigated in this study. While all five P2X ionotropic purinergic receptor subtypes were confirmed, only $P2Y_1$ and $P2Y_4$ could be identified, and the $P2Y_2$ receptor protein was weakly expressed in total cell lysates of CPCs.

To rule out the function of certain P2X and P2Y receptors, the nonspecific P2 receptor antagonist suramin (10 μM) was administered prior to and during local application of P2 receptor agonists. None of the parameters of Ca^{2+} transients evoked either in 1.8 mM or 0 mM Ca^{2+}-containing Tyrode's were altered by the presence of suramin, indicating that mainly suramin-insensitive P2 purinergic receptors (*i.e.* $P2X_4$, $P2X_6$, and/or $P2Y_4$) were responsible for the observed effects.

Autocrine/paracrine purinergic signalling is required for repetitive Ca^{2+} transients in CPCs

When apyrase, an enzyme that catalyses the hydrolysis of ATP to AMP and inorganic phosphate, was administered to cells prior to and during single cell Ca^{2+} measurements at 5 $U \cdot mL^{-1}$ concentration, no repetitive Ca^{2+} transients were observed (n=5), implicating the requirement of extracellular ATP for the initiation of Ca^{2+} oscillations in CPCs.

Results II.

Cell culturing and transfection

The effect of SERCA1b silencing was thus examined in a mouse skeletal muscle cell line. SERCA1b protein synthesis has been interfered with using a specific shRNA sequence cloned into pLKO.1-puro-CMV-tGFP expression vector. Decreased protein expression was confirmed in the selected clones using a SERCA1b specific antibody at the myotube stage. According to our observations the SERCA1 positivity was similar to SERCA1b expression pattern. Quantitative analysis of the Western-blots confirmed a very pronounced decrease in SERCA1b expression in certain identified clones compared to that of scrambled shRNA transfected cells. CloneC1 and another clone (C5) – in

which SERCA1b was downregulated to a lesser extent – were selected for further experiments. Scrambled shRNA transfected cells were used as a control. All the experiments were performed on multinucleated, terminally differentiated myotubes on the 5th day of differentiation.

Reduced SERCA1b expression alters the expression of proteins involved either in the Ca^{2+}-homeostasis or differentiation of skeletal muscle

Next the expression level of calsequestrin (CSQ) – the main Ca^{2+}-binding protein–and the stromal interacting molecule1 (STIM1) – the calcium sensor of SOCE in the SR – were studied by Western-blot analysis. The scrambled shRNA transfection did not modify the expression level of CSQ, while in the cloneC5 myotubes the expression showed a moderate decrease. Furthermore, in cloneC1 cells only a very weak band could be detected. Similarly, STIM1 expression was slightly reduced in scrambled shRNA transfected and cloneC5 cells as compared to the parental cells, while the STIM1 expression was hardly detectable in cloneC1. Expression pattern of the main regulatory proteins that play an essential role in skeletal muscle differentiation were examined by Western-blotting and RT-PCR analysis. MyoD, and CaN were clearly detectable at protein level. The expression of the myogenic transcription factor (MyoD) was found to be unaffected. The CaN showed a remarkable decrease in cloneC1 and even in cloneC5, as compared to control cell types. Quantitative analysis of the results after normalizing to actin also confirmed the significant alterations in CSQ, STIM1, and CaN expression detected in cloneC1 as compared to control cells. DHPR expression was similar in transfected and parental C2C12 cells. Using specific SERCA2a antibody was detected with similar intensity at ~115 kDa in all C2C12 types.

Using specific primer pairs, myostatin showed a significantly decreased mRNA expression in cloneC1 as compared to scrambled shRNA transfected cells, thus the myostatin transcript level correlated with the SERCA1b silencing.

In parallel MCIP1.4 was proved to be statistically modified in cloneC1. The application of a CaN activity assay revealed the significantly reduced activity of CaN in line with its decreased expression.

Effect of reduced SERCA1b expression, on Ca^{2+}-homeostasis

To examine the possible alteration of E-C coupling, repeated Ca^{2+}-transients were evoked by the applications of 120 mM KCl in scrambled shRNA transfected and cloneC1 myotubes. Neither the amplitude of the transients (681±105, n=10 *vs*. 668±128 nM, n=10 in control and in cloneC1 cells, respectively) nor their rate of rise (606±108 μM/s *vs*. 489±149 μM/s, respectively) were significantly altered ($p>0.5$) in cloneC1 myotubes.

To analyze the functional effects of decreased SERCA1b expression, the return of $[Ca^{2+}]_i$ to its resting value following the KCl-evoked transients and the maximal transport rate of the Ca^{2+} pump (PV_{max}) were compared in scrambled shRNA transfected and cloneC1 and C5 myotubes. There was no difference in the resting $[Ca^{2+}]_i$ before the transients (108±4 nM in scrambled shRNA transfected and 109±4 nM in cloneC1 myotubes). Following the KCl-evoked transients $[Ca^{2+}]_i$ declined slower and returned to a significantly higher level in the clone C1 myotubes. A significantly higher $[Ca^{2+}]_i$ could be measured 20 and 40 seconds after the beginning of KCl application (112±3 nM, and 110±3 in control, while 150±7 nM and 135±5 in cloneC1 cells, respectively) indicating a decreased Ca^{2+}-uptake capability of the SERCA pumps. This was further quantified by extracting PV_{max}(maximal transport rate of the pump), which was clearly decreased ($p<0.01$) in cloneC1 myotubes (144±24 μM/s) and in cloneC5 myotubes (382±16 μM/s) as compared to scrambled shRNA transfected cells (454±41 μM/s) in line with the decreased expression of SERCA.

In addition a slight reduction in the maximal rate-of-rise of KCl-evoked calcium transients was also observed. These findings can only be reconciled if the

amount of calcium released from the SR was reduced in cloneC1 cells. The calcium flux (610±60 *vs.* 377±64 μM/s, respectively) were suppressed significantly (*p<0.01*) as a result of SERCA1b silencing. The calculated integral for the releasable calcium was also proved to be significantly lower in cloneC1 myotubes as compared to scrambled shRNA transfected cells (843±75 μM *vs.* 576±80 μM; *p<0.02*). These observations suggest that the Ca^{2+}-content of the SR and, consequently, the rate of Ca^{2+}-release into the cytosol was decreased when the SERCA1b expression was interfered with. When the SERCA blocker CPA (10 μM) was administered to cells in Ca^{2+} free Tyrode's, a pronounced elevation in resting Ca^{2+} levels was observed, caused by Ca^{2+} release from the SR *via* RyRs and concurrent inhibition of Ca^{2+} re-uptake. The releasable Ca^{2+} content of internal Ca^{2+} stores could be calculated, which was significantly lower in C1 cells compared to scrambled shRNA transfected myotubes (5.8±1.1 μM*s and 15.7±1.8 μM*s; $p<0.001$).

SERCA1b silencing modifies the growth of differentiating skeletal muscle

Morphological alterations of the cells in culture with decreased SERCA1b expression were also observed during the proliferation and differentiation period. Multinucleated myotubes were treated with anti-desmin primary antibody and visualized with FITC conjugated secondary antibody. The diameter of terminally differentiated cloneC1 myotubes did not differ significantly ($p>0.2$) from that of the control. In contrast, the average number of nuclei was significantly decreased (5.7±0.5 in scrambled shRNA transfected cells and 3.6±0.2 in cloneC1 cells, $p<0.01$). Myotubes containing 5 or more nuclei could be detected in a lower ratio than it was observable in control cultures. To investigate the proliferation rate, 10,000 cells were plated on the 0^{th} day of culturing. On the 4^{th} day of culturing the rate of proliferation was significantly lower ($p<0.05$) in the cloneC1 cultures, as compared to parental cells (3.7±1.1 and 9.1±0.7, respectively).

Since the expression and activity of CaN was found to be reduced in cloneC1 myotubes, the effect of CSA was investigated on the rate of proliferation of C2C12 cells. CSA significantly inhibited the proliferation of parental cells as indicated by the relative number of myogenic nuclei on the 4^{th} day (9.1±0.7 *vs*.5.6±0.6; in control and CSA treated cells, respectively; $p<0.05$). On the other hand, CSA did not further decrease the rate of proliferation in cloneC1 cultures (3.6±0.5 *vs*. 3.7±1.1; in CSA treated and non-treated cloneC1 cells, respectively; $p>0.4$).

Discussion I.

Dynamics of cytosolic $[Ca^{2+}]_i$

Ca^{2+} oscillations, represent a nearly universal signalling mechanism in non-excitable cells by reducing the threshold for the activation of NFAT, NF-κB or CREB and by modulating protein kinase activity. This is the first study to report that CPCs are also characterised by a similarly dynamic Ca^{2+} homeostasis. CPCs exhibited periodic increases in $[Ca^{2+}]_i$, Ca^{2+} oscillations in CPCs were even less frequent (1.3×10^{-3} Hz).

Functional characterisation of internal Ca^{2+} stores and their role in generating Ca^{2+} oscillations

Next, we analysed the presence and function of ER-related Ca^{2+}-signalling mechanisms. CPCs express all three IP_3R subtypes. In fact, the properties of Ca^{2+} oscillation patterns depend on the relative expression levels of IP_3R subtypes. The application of the aspecific IP_3R blocker 2-APB during single cell Ca^{2+} measurements completely abolished spontaneous Ca^{2+} events. This might be a universal mechanism in the generation of Ca^{2+} oscillations in stem cells.

The claim that Ca^{2+} oscillations in CPCs were predominantly dependent on Ca^{2+} release through IP_3R followed by store replenishment *via* SOCE is

supported by key data showing that the amount of Ca^{2+} liberated during spontaneous Ca^{2+} oscillations (23.6±5.4 μM · s) was almost identical to Ca^{2+} released during CPA-induced store depletion (22.2±2.4 μM · s). The CPCs express only the STIM1 subtype may have important functional consequences for STIM2 is known to respond to smaller decreases in the ER Ca^{2+} content. STIM1 only becomes active once the stores are almost fully emptied. As far as Orai isoforms are concerned, we confirmed that CPCs express Orai1 proteins only.

Purinergic signalling in CPCs and autocrine/paracrine regulation of Ca^{2+} oscillations

We aimed to study whether CPCs were able to respond to nucleotide stimulation by P1 and/or P2 receptor activation. Purinergic receptors are conventionally classified as adenosine or P1 receptors, and P2 receptors, primarily recognizing ATP, ADP, UTP, and UDP.

First, we established the P1 purinergic receptor profile of CPCs. The P1 receptor subtypes were found to be expressed in articular chondrocytes. Next, we analysed the P2 purinergic receptor expression in CPCs. Since Ca^{2+} transients elicited by extracellular ATP administration during fluorescent single cell Ca^{2+} measurements were significantly smaller in medium with 0 mM $[Ca^{2+}]_e$ compared to 1.8 mM $[Ca^{2+}]_e$ solution, the involvement of both P2X and P2Y receptor isotypes could be implicated. By contrast, when ATP was co-applied with the IP_3R inhibitor 2-APB, the majority of cells was unresponsive, suggesting that the primary mediators of extracellular nucleotides were metabotropic P2Y receptors. Therefore, using Western blotting, we confirmed the presence of both P2X and P2Y receptor proteins.

To narrow down functional P2Y receptors, ADP, UTP and UDP were administered to CPCs during Ca^{2+} measurements. In this study, only the G_q-

coupled P2Y receptors (*i.e.* $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) were studied. Based on their agonist specificities, ADP may primarily act on $P2Y_1$; $P2Y_4$ may be the primary target of UTP; and ATP may elicit Ca^{2+} transients *via* all detected P2X subtypes, as well as $P2Y_1$ and $P2Y_2$. By using suramin as a non-specific P2 receptor antagonist that inhibits P2 receptor activity with the exemption of $P2X_4$, $P2X_6$, as well as $P2Y_4$ and $P2Y_6$, ATP could still evoke Ca^{2+} transients, suggesting the active function of $P2X_4$ and $P2X_6$ receptor subtypes. In this work, we propose that purinergic signalling may be the main drive Ca^{2+} oscillations in CPCs as apyrase was able to completely abrogate Ca^{2+} oscillations, and the very same P2 purinergic receptors (*i.e.* $P2X_4$, $P2Y_1$) known to be key regulators of ATP-driven Ca^{2+} oscillations in MSCs and chondroprogenitor cells were functional also in these cells.

Discussion II.

Effects of SERCA1b silencing on Ca^{2+}-homeostasis

The present work provides the first clear evidence that the sequence spliced together from exon 21 and 23 of the SERCA1 transcript can be targeted for efficient silencing of the neonatal SR Ca^{2+}-pump protein in myogenic cells. We established the level and activity of the calcium-dependent CaN was also decreased even in a less (C5) not just in the most silenced clone (C1). However, the MCIP1.4 mRNA level, declined only in the most effectively SERCA1b silenced (cloneC1) myotubes indicating an indirect and less prompt response than CaN. CSQ declined only in the most silenced clone (C1) implicating a lower need of binding SR Ca^{2+}.

As we pointed out earlier, the SERCA1b isoform is probably responsible for most of the SERCA1. STIM1 binds to SERCA1a and stimulates its activity in rabbit skeletal muscle and in primary mouse myotube. Such binding can only happen to a peptide sequence that must also be present in SERCA1b since

SERCA1b contains the entire sequence of SERCA1a except the C-terminal glycine. Generating an antibody is not feasible against a C-terminal glycine since it can be present in many other proteins besides SERCA1a. Therefore might well have demonstrated the STIM1 effect on SERCA1b, because they worked with primary mouse myotubes that probably have SERCA1b as the main SERCA1 isoform. Anyway the present work is in agreement with the importance of SERCA1b in SOCE because the level of the calcium sensor STIM1 was decreased in SERCA1b silenced myotubes. It is a general observation that elements of SOCE are regulated by a positive feedback during myotube development. Therefore, feasible to suggest that the lower level of STIM1 and SERCA1b generates lower rate of Ca^{2+} movement across the myoplasm which in turn stimulates less calcineurin and results in reduced downstream gene activity reported by the MCIP1.4 mRNA level. Similarly, the silenced SERCA1b could fill the SR to a lower Ca^{2+} level requiring, therefore, a lesser amount of CSQ for storage. On the other hand, the silencing did not modify the myogenic transcription factor MyoD level. This is in agreement with the observation that the expression of MyoD is started earlier, than that of SERCA1b during the differentiation of C2C12 cells.

Functional characterisation of SERCA1b silencing myotubes

To examine the effects of SERCA1b silencing on excitation-contraction coupling, repeated Ca^{2+} transients were evoked. Neither the amplitude nor the rate of raise of the transients in cloneC1 myotubes was different from those measured in the scrambled control cells indicating that the function of RyR and the voltage-gated channels was not likely affected. Exposure to high K^{+} concentration triggered similar Ca^{2+} elevations in the sarcoplasmic space of both SERCA1b silenced and non-silenced myotubes. Noteworthy in this context that the calcium was evidently reuptaken into the SR even in the SERCA1b silenced myotubes indicating that other SERCA pumps also take part in this process. The

SERCA1b silenced myotubes restored the sarcoplasmic calcium back to the normal level much slower.

On the other hand, the relatively normal Ca^{2+} elevation in the sarcoplasm and the slower Ca^{2+} removal from the sarcoplasm in silenced myotubes could not be reconciled without assuming a decreased amount of released Ca^{2+} into the sarcoplasmic space and without a lower Ca^{2+} influx, which was indeed found. These parameters together enlighten a lower releasable SR Ca^{2+} content in silenced than in control myotubes. This suggests that SERCA1b significantly contributes to the refilling of the SR with normal amounts of calcium. It is noteworthy in this context that 1-5 hours after birth, when SERCA1 knock-out mice usually die, SERCA1b is still the dominantly expressed isoform in the corresponding muscles of wild type mice. The changes in the Ca^{2+} homeostasis of SERCA1b silenced C2C12 cells is also in agreement with the suggestion that the lack of the neonatal Ca^{2+} pump might be responsible for the effects seen in SERCA1 KO mice. Cell proliferation is sensitively connected to SOCE and SERCA. In accordance, the more (cloneC1) and even the less (cloneC5) efficiently silenced cells showed inhibited muscle cell proliferation and fewer myogenic nuclei in their cultures as compared to that of control cells. The necessity of SERCA1b for cell proliferation seems to be connected CaN regulated signaling pathway since CSA, a calcineurin inhibitor, decreased cell proliferation in the parental non-transfected cells, but surprisingly no further decrease of proliferation was observed in the most silenced clone (cloneC1). Our finding that CSA did not inhibit the proliferation in SERCA1b silenced myogenic cell lines suggests that either the CSA-dependent component of calcineurin activity was mostly based on SERCA1b activity or raise the possibility of a calcineurin independent CSA effect.

Morphological alterations of the cells in culture with decreased SERCA1b expression

Difference between myotube nuclear numbers of the most silenced cell line and of the one transfected with scrambled oligos was also significant. Myotube formation is based on the density of myoblasts in a certain field. First the primary myotubes are formed with 4-6 nuclei then the secondary myotubes develop further accumulating nuclei from fusing myoblasts preferably at their end. SERCA1b silenced myotubes (cloneC1) show a strong incline of having less than five nuclei. The result also demonstrates that SERCA2a alone is not able to compensate completely for the declined (cloneC5) or abolished (cloneC1) SERCA1b in myoblast proliferation, only for the SERCA1b decline (cloneC5) in myotube development. In summary, SERCA1b is required for myoblast proliferation and secondary myotube formation in murine C2C12 myogenic cells by having an important role in Ca^{2+} homeostasis. During this process its expression is coupled to those of STIM1, CSQ, and calcineurin suggesting a role in SOCE, too.

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List of publications related to the dissertation

1. **Tóth, A.**, Fodor, J., Vincze, J., Oláh, T., Juhász, T., Zákány, R., Csernoch, L., Zádor, E.: The Effect of SERCA1b Silencing on the Differentiation and Calcium Homeostasis of C2C12 Skeletal Muscle Cells.
   PLoS One. 10 (4), 25 p., 2015.
   DOI: http://dx.doi.org/10.1371/journal.pone.0123583
   IF:3.534 (2013)

2. Matta, C., Fodor, J., Miosge, N., Takács, R., Juhász, T., Rybaltovszki, H., **Tóth, A.**, Csernoch, L., Zákány, R.: Purinergic signalling is required for calcium oscillations in migratory chondrogenic progenitor cells.
   Pflugers Arch. 467 (2), 429-442, 2015.
   DOI: http://dx.doi.org/10.1007/s00424-014-1529-8
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List of other publications

3. Nagy, D., Gönczi, M., Dienes, B., Szöőr, Á., Fodor, J., Nagy, Z., **Tóth, A.**, Fodor, T., Bai, P., Szűcs, G., Rusznák, Z., Csernoch, L.: Silencing the KCNK9 potassium channel (TASK-3) gene disturbs mitochondrial function, causes mitochondrial depolarization, and induces apoptosis of human melanoma cells.
Arch. Dermatol. Res. 306 (10), 885-902, 2014.
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Cell Calcium. 54, 1-16, 2013.
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