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Store-operated calcium entry and calcium influx *via* voltage-operated calcium channels regulate intracellular calcium oscillations in chondrogenic cells

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ABSTRACT

Chondrogenesis is known to be regulated by calcium-dependent signalling pathways in which temporal aspects of calcium homeostasis are of key importance. We aimed to better characterise calcium influx and release functions with respect to rapid calcium oscillations in cells of chondrifying chicken high density cultures. We found that differentiating chondrocytes express the α_1 subunit of voltage-operated calcium channels (VOCCs) at both mRNA and protein levels, and that these ion channels play important roles in generating Ca^{2+} influx for oscillations as nifedipine interfered with repetitive calcium transients. Furthermore, VOCC blockade abrogated chondrogenesis and almost completely blocked cell proliferation. The contribution of internal Ca^{2+} stores *via* store-operated Ca^{2+} entry (SOCE) seems to be indispensable to both Ca^{2+} oscillations and chondrogenesis. Moreover, this is the first study to show the functional expression of STIM1/STIM2 and Orai1, molecules that orchestrate SOCE, in chondrogenic cells. Inhibition of SOCE combined with ER calcium store depletion abolished differentiation and severely diminished proliferation, suggesting the important role of internal pools in calcium homeostasis of differentiating chondrocytes. Finally, we present an integrated model for the regulation of calcium oscillations of differentiating chondrocytes that may have important implications for studies of chondrogenesis induced in various stem cell populations.

1. Introduction

One of the initial steps during formation of the embryonic skeleton is the differentiation of chondroprogenitor cells into extracellular matrix-secreting chondroblasts. Later on, mature chondrocytes are formed that undergo terminal differentiation preceding endochondral ossification [1]. Cartilage formation, including differentiation and proliferation of chondrogenic cells, is tightly regulated by complex interplay between numerous families of signalling proteins, eventually leading to activation of Sox9, as well as Sox6 and L-Sox5—transcription factors that are essential for chondrogenesis; they are involved in the specification of the chondrogenic lineage and activate the expression of chondrogenic marker genes (*e.g. COL2A1*, *AGR1*) [2,3].

During the early steps of chondrogenesis, intracellular signalling undergoes profound changes to initiate specific gene activation that requires translocation of lineage-specific transcription factors into the cell nucleus. In particular, the nuclear localisation signal at the N-terminus of the chondrocyte-specific transcription factor Sox9 was shown to contain a calmodulin-binding region and Ca^{2+}–calmodulin has been reported to be involved in the nuclear entry of Sox9 [4]. Calcium ions (Ca^{2+}) are suggested to be key

Abbreviations: AM, acetoxy-methylester; ARC, arachidonate-regulated Ca^{2+}-sensitive channel; ATP, adenosine triphosphate; CaMKII, Ca^{2+}–calmodulin dependent protein kinase II; CPA, cyclopiazonic acid; CRAC, Ca^{2+} release-activated Ca^{2+} channel; CREB, cAMP-response element binding protein; DMMB, dimethylmethylene blue; ECM, extracellular matrix; ER, endoplasmic reticulum; FBS, foetal bovine serum; FTHM, full time at half maximum; HDC, high density cell culture; HRP, horse radish peroxidase; IP_3, inositol-1,4,5-trisphosphate; IP_3R, inositol-1,4,5-trisphosphate receptor; MSC, mesenchymal stem cell; NCX, Na^+–Ca^{2+} exchanger; NFAT, nuclear factor of activated T lymphocytes; NMDA, *N*-methyl-D-aspartate receptor; PKC, protein kinase C; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+}-ATPase; RMP, resting membrane potential; RT-PCR, reverse transcription followed by polymerase chain reaction; RyR, ryanodine receptor; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SOC, store-operated calcium channel; SOCE, store-operated Ca^{2+} entry; STIM, stromal interaction molecule; TB, toluidine blue; TRP, transient receptor potential receptor; TRPC, canonical transient receptor potential receptor; TRPV, transient receptor potential receptor vanilloid; VOCC, voltage-operated calcium channel.

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factors involved in controlling differentiation of cells, including human mesenchymal stem cells (MSCs) [5] and chondrocytes [6].

In general, Ca^{2+}-regulated cellular events require transiently elevated cytosolic Ca^{2+} concentration to activate Ca^{2+}-sensitive signalling components [7]. Cells utilise two main sources of Ca^{2+} for initiating and generating signals. On the one hand, Ca^{2+} entry across the plasma membrane *via* either voltage-operated Ca^{2+} channels (VOCCs) or agonist-dependent and voltage-independent Ca^{2+} entry pathways, amongst which are the receptor ion channels (*e.g.* P2X [8], NMDA-receptors [9], TRPV channels [10]; the latter group also act as sensors of osmotic pressure, volume, stretch, and various chemical and mechanical stimuli [11]), the store-independent (*e.g.* arachidonate-dependent non-capacitive Ca^{2+} entry [12]) and 'store-operated' Ca^{2+} (SOC) channels (*e.g.* TRPs [5]); and on the other hand, Ca^{2+} release from internal stores such as the smooth endoplasmic reticulum (ER) *via* the inositol-1,4,5-trisphosphate receptor (IP_3R) or the ryanodine receptor (RyR), as well as Ca^{2+} induced Ca^{2+} release (CICR) or *via* the exchange protein directly activated by cyclic AMP (Epac) [13,14]. Since changes of intracellular Ca^{2+} concentration are related to cell proliferation and differentiation that are important functions of undifferentiated cells such as MSCs, the Ca^{2+} homeostasis of stem cells has been thoroughly investigated [15].

It is well documented that different spatial and temporal patterns of intracellular free Ca^{2+} concentration play distinct roles in the regulation of various cellular processes. Not only a stable rise, but also periodic oscillatory changes of cytosolic Ca^{2+} concentration represent a nearly universal signalling mechanism even in non-excitable cells [16]. Signal transduction pathways triggered by Ca^{2+} oscillations are generally accepted to control cellular differentiation *via* reducing the threshold for the activation of different Ca^{2+}-dependent transcription factors, including nuclear factor of activated T lymphocytes (NFAT), nuclear factor-κB (NF-κB), Jun N-terminal kinase-1 (JNK1), myocyte enhancer factor-2 (MEF2) and the cAMP-response element binding protein (CREB), depending on the frequency and amplitude of the Ca^{2+} transients [17,18]. Spontaneous Ca^{2+} oscillations were reported in several non-excitable cells undergoing differentiation or proliferation [19]. In human MSCs, the major source of Ca^{2+} for the observed oscillations is Ca^{2+} release from ER *via* IP_3Rs, but Ca^{2+} influx *via* non-capacitive SOCs is also required to sustain these oscillations, without a significant contribution from VOCCs [5]. Unlike MSCs, spontaneous Ca^{2+} oscillations in most of the cells examined, including differentiating limb bud-derived mesenchymal cells, seemed to depend on the availability of extracellular Ca^{2+}. These observations are in a good correlation with our previous results implying that mainly Ca^{2+} influx, at least in part *via* the ligand-gated purinergic cation channel $P2X_4$, is required for maintaining sustained raised cytosolic Ca^{2+} levels in differentiating chondrogenic cells [20,21]. Nevertheless, in these studies, the contribution from internal Ca^{2+} stores, in spite of the functional expression of IP_3Rs, seemed to be less important in differentiating chicken chondrogenic cells. Many Ca^{2+} entry and release processes have been documented in mature chondrocytes [22]; however, knowledge regarding the precise regulation and function of Ca^{2+} homeostasis, including high-frequency spontaneous oscillations, during *in vitro* chondrogenesis is still sparse.

In this study, we aimed at further characterising the Ca^{2+} homeostasis of differentiating chicken chondrocytes with special emphasis on the contribution of internal Ca^{2+} stores and the involvement of VOCCs in generating and maintaining high-frequency repetitive Ca^{2+} transients to modulate cellular functions such as differentiation and proliferation. We undertook to analyse intracellular Ca^{2+} dynamics in individual chondrifying cells at high spatial and temporal resolution using LIVE confocal Ca^{2+} imaging microscopy. Having performed a detailed investigation of the contribution of internal Ca^{2+} stores and store-operated Ca^{2+} entry (SOCE) to the Ca^{2+} homeostasis of differentiating chondrocytes and the involvement of Ca^{2+} influx *via* VOCCs in generating or maintaining spontaneous Ca^{2+} oscillations, we provide a refined model of Ca^{2+} signalling events including Ca^{2+} influx and release functions in differentiating cells of chondrifying micromass cultures during *in vitro* chondrogenesis.

2. Materials and methods

2.1. Primary high density chondrifying cell cultures

A well-known and easily reproducible *in vitro* experimental model to study hyaline cartilage formation was first described by Ahrens et al. [23]. In these high density cell cultures (HDC), chicken limb bud-derived chondroprogenitor mesenchymal cells spontaneously differentiate to chondroblasts and chondrocytes on days 2 and 3 of culturing, and a well-detectable amount of hyaline cartilage extracellular matrix (ECM) is produced by day 6.

To establish primary micromass cell cultures of chondrifying mesenchymal cells, Ross hybrid chicken embryos of Hamburger–Hamilton developmental stages 22–24 (4.5-day-old) were used. Work on early chick embryos *in vitro* does not require a license from the Ethics Committee of the University of Debrecen. Distal parts of forelimbs and hindlimbs of embryos were isolated and dissociated in 0.25% trypsin–EDTA (Sigma, St. Louis, MO, USA; pH 7.4) at 37 °C for 1 h. The enzymatic digestion was terminated by the addition of equal volume of foetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and digested limb buds were filtered through a 20-μm pore size plastic filter unit (Millipore, Billerica, MA, USA) to yield a single cell suspension of chondrogenic mesenchymal cells. After a brief centrifugation (at 800 × *g* for 10 min), cells were resuspended in Ham's F12 medium (Sigma) supplemented with 10% FBS at a concentration of 1.5×10^7 cells mL^{-1} and 100–100 μL droplets were inoculated into plastic cell culture plates (Orange Scientifique, Braine-l'Alleud, Belgium). After allowing the cells to attach to the surface for 120 min at 37 °C in a CO_2 incubator (5% CO_2 and 80% humidity), 2 mL of Ham's F12 supplemented with 10% FBS, 0.5 mM stabile L-glutamine and antibiotics/antimicotics (penicillin 50 U mL^{-1}, streptomycin 50 μg mL^{-1}, fungizone 1.25 μg mL^{-1}; TEVA, Debrecen, Hungary) was added. Day of inoculation was considered as day 0 of culturing. Cultures were kept at 37 °C in a CO_2 incubator for 6 days. The medium was changed on every second day.

2.2. Confocal microscopy

2.2.1. Line-scan analysis

Spontaneous Ca^{2+} transients and the effects of modified extracellular ionic milieu or various drugs on Ca^{2+} oscillations were monitored using an LSM 510 META Laser Scanning Confocal Microscope (Zeiss, Oberkochen, Germany). All measurements were performed at room temperature. Cells of 1- and 2-day-old high density micromass cell cultures were incubated for 30 min at 37 °C with 10 μM Fluo-4-AM in Ham's F12 medium. Calcium imaging was carried out in normal (in mM: 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 11.8 HEPES; 1 g L^{-1} glucose; pH 7.4) or Ca^{2+}-free (containing 5 mM EGTA, without $CaCl_2$) Tyrode's solution. The sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA, Sigma) was used at a final concentration of 10 μM in normal Tyrode's solution (stock: 10 mM, in DMSO). SOCE blockers, *i.e.* the non-specific TRPC antagonist YM-58483 (a pyrazole derivative, also known as BTP-2; Sigma) [24] and $LaCl_3$ (Sigma) [5] were used at 1 μM and 500 μM final concentrations, respectively, diluted in normal Tyrode's solution (stocks: 300 mM and 1 mM in distilled water and DMSO, respectively).

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(These compounds could not be applied to oscillating cells in Ca^{2+}-free Tyrode's since removal of free extracellular Ca^{2+} ions alone abolished Ca^{2+} oscillations, which would have rendered the evaluation of the effects of these blockers impossible.) Acquisition of line-scan images started immediately after changing the solution on the cultures. During measurements, only cells exhibiting Ca^{2+} oscillations were investigated, and other cells were disregarded. Line-scan images were acquired at 0.8 ms/line, 512 pixels/line with 7 ms intervals, recording 8192 lines using a 63× water immersion objective. Measurements were carried out in cells from 3 independent experiments. Images were analysed using an automatic event detection software developed in the Department of Physiology of the University of Debrecen, Medical and Health Science Centre.

2.2.2. X–Y monitoring

Series of X–Y images were recorded from random visual fields of 1- and 2-day-old Fluo-4-loaded chondrifying cultures with LIVE 5 Laser Scanning Confocal Microscope (Zeiss, Oberkochen, Germany) using EC Plan-Neofluar 20×/0.50 M27 objective with 2× digital zoom. Calcium imaging was performed in normal and Ca^{2+}-free Tyrode's solutions (see above). All measurements were performed at room temperature. $LaCl_3$ (500 μM), YM-58483 (1 μM), and nifedipine (10 μM) were diluted in normal Tyrode's solution (containing 1.8 mM Ca^{2+}; see above). Frame acquisition rate was 10 s^{-1}. A total number of 1000 images were recorded during control conditions on days 1 and 2. When the effects of pharmacons on spontaneous Ca^{2+} transients were examined during time series recordings, the same visual fields on each culture were observed 1, 3 and 5 min after replacing the bath solution from normal Tyrode's to the test solution ($LaCl_3$–YM-58483; or nifedipine). During these latter experiments, a total number of 500 X–Y scans were recorded at each time point. Data analysis was carried out using Zeiss Enhanced Navigation (ZEN 2009) software. The round-shaped chondrocytes were marked as region of interest (ROI) on each visual field. Time-dependent fluorescent intensities of ROIs were analysed using an automatic event detection software developed in the Department of Physiology.

2.3. Single cell fluorescent Ca^{2+} measurements

Measurements were performed on day 2 of culturing using the calcium dependent fluorescent dye Fura-2 as described previously [6]. Briefly, cultures were transferred to 2 mL fresh Ham's F12 medium containing 10 μL Fura-2-acetoxy-methylester (AM; 10 μM) and 4 μL neostigmin (0.3 nM; to inhibit extracellular choline-esterase activity). Fura-2-loaded cells were then placed on the stage of an inverted fluorescent microscope (Diaphot; Nikon, Kowasaki, Japan) and viewed using a 40× oil immersion objective. Measurements were performed in normal and Ca^{2+}-free Tyrode's solutions (see above). $LaCl_3$ (500 μM), YM-58483 (1 μM), and CPA (10 μM) were diluted in Ca^{2+}-free Tyrode's solution. Excitation wavelength was altered between 340 and 380 nm (F_{340} and F_{380}) by a microcomputer-controlled dual-wavelength monochromator (DeltaScan; Photon Technologies International, New Brunswick, NJ, USA). Emission was monitored 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 et al. [25]. The measuring bath was constantly perfused with normal Tyrode's solution at a rate of 2 mL min^{-1} (EconoPump; Bio-Rad Laboratories, CA, USA). Test solutions were directly applied to the cells through a perfusion capillary tube (Perfusion Pencil™; AutoMate Scientific, San Francisco, CA, USA) with an internal diameter of 250 μm at a rate of 1.5 μL s^{-1}, using a local perfusion system (Valve Bank™ 8 version 2.0, AutoMate Scientific). All measurements were performed at room temperature.

2.4. Modulation of Ca^{2+} influx via voltage-operated Ca^{2+} channels with nifedipine and inhibition of SOCE

In order to assess longer-term effects of interference with Ca^{2+} homeostasis of differentiating chondrocytes, the above compounds were also added to the culture medium of HDC. The L-type VOCC-blocker nifedipine was administered to the culture medium from the beginning of the first culturing day at a final concentration of 10 μM. For control experiments, the vehicle (DMSO, Sigma) was added to cultures at equal volumes. To assess the role of internal Ca^{2+} stores, following store depletion with 10 μM CPA, SOCE was blocked by co-application of 1 μM YM-58483 and 500 μM $LaCl_3$. CPA, YM-58483 and $LaCl_3$ were added to the culture medium on culturing day 2 for 24 h. For control experiments, the vehicles (DMSO and sterile water) were added to cultures at equal volumes.

2.5. Qualitative and semi-quantitative determination of cartilage matrix production

For visualisation of cartilage matrix in HDC, low pH metachromatic staining was performed with dimethyl methylene blue (DMMB; Sigma) dissolved in 3% acetic acid on day 6 of culturing. The amount of sulphated matrix components was determined with a semi-quantitative method, by measuring the optical density of extracted toluidine blue (TB; Reanal, Budapest, Hungary) bound to glycosaminoglycans in 6-day-old HDC. Both qualitative and semi-quantitative staining procedures were described previously in more detail [6].

2.6. Measurement of cell proliferation and mitochondrial activity

Rate of cellular proliferation in HDC was determined by measuring the radioactivity of incorporated ^{3}H-thymidine during a 16-h-long period on day 3 (as described earlier [6]) started promptly after combined treatment with CPA, YM-58483 and $LaCl_3$, or nifedipine. For the investigation of mitochondrial activity, cells cultured in wells of 96-well plates were used and MTT-assay was performed immediately after treatments on day 3 as it was described previously [6]. Untreated 3-day-old HDC were used as controls for both assays. Measurements were carried out in 6 samples of each experimental group in 3 independent experiments.

2.7. Reverse transcription followed by PCR analysis

Total RNA from HDC was isolated as described previously [6]. The assay mixture (20 μL) for reverse transcriptase (RT) reactions contained 500 ng total RNA, 0.25 μL RNase inhibitor, 2 μL random primers, 0.8 μL dNTP Mix (4 mM), 50 units (1 μL) MultiScribe™ RT in 1× RT buffer (High Capacity RT kit; Applied Biosystems, Foster City, CA, USA) and complementary cDNA was transcribed at 37 °C for 2 h. Amplifications of specific cDNA sequences were achieved with specific primer pairs that were designed based on chicken nucleotide sequences published in GenBank and purchased from Integrated DNA Technologies, Inc. (IDT; Coralville, IA, USA). Nucleotide sequences of forward and reverse primers and reaction conditions are shown in Table 1. 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) Promega GoTaq® DNA polymerase in 1× Green GoTaq® Reaction Buffer in a programmable thermal cycler (Labnet MultiGene™ 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) with the following settings: 2 min at 95 °C

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Table 1
Nucleotide sequences, amplification sites, GenBank accession numbers, amplicon sizes and PCR reaction conditions for each primer pair are shown.

Gene	Primer	Nucleotide sequence (5′ → 3′)	GenBank ID	Annealing temperature	Amplicon size (bp)
$Ca_V1.2$ (CACNA1C)	Sense Antisense	CAA CAG AGC CAA AGG ACT AAA (3054–3074) GTG ACG ATG ACG AAA CCA A (3512–3530)	XM_416388	54 °C	477
$Ca_V1.3$ (CACNA1D)	Sense Antisense	AGG CTC ATC AAT CAC CAC A (2704–2722) AAA GAC GCA CTG AAC AAC G (3072–3090)	NM_205034	54 °C	387
$Ca_V2.2$ (CACNA1B)	Sense Antisense	CTA CGC CAC GAC CCT ACA C (2442–2460) TTC TCA ACG CCT TCT TCC A (2831–2849)	NM_204293	61 °C	408
$Ca_V2.3$ (CACNA1E)	Sense Antisense	TCA CCA ACT CCG ACC GTA AC (3347–3366) CAC CTC CAT CTT GTT CTT CTC AT (3824–3846)	XM_422255	60 °C	500
$Ca_V3.1$ (CACNA1G)	Sense Antisense	CAC TGA ATC CGT CCA TAG CAT C (1989–2010) CTG TCT GAG TCC GTC TCG TTG T (2390–2411)	XM_001232653	61 °C	423
$Ca_V3.2$ (CACNA1H)	Sense Antisense	CCC TGG AAG GAT GGG TTG A (1256–1274) CTG CCC GTT TGT GGT GTT G (1608–1626)	XM_414830	61 °C	371
$Ca_V3.3$ (CACNA1I)	Sense Antisense	CTG AGG ACG GAT ACA GGA GAT (2281–2301) TTG CGT GAA GAG TTG GAG AC (2698–2717)	XM_425474	59 °C	437
Orai1	Sense Antisense	TAG CAA CGT GCA TAA TCT CAA (264–284) TCA GTC CAA AGG GAA CCA T (502–520)	NM_001030658	57 °C	257
STIM1	Sense Antisense	GGT GGT GTC CAT CGT CAT CG (426–445) GCT CCT TCT CGG CGT TCT TC (762–781)	NM_001030838	62 °C	356
STIM2	Sense Antisense	CAA TTA GCA ATC GCC AAA G (1177–1195) CAC AGA AAG GAT GTC AGG GT (1652–1671)	XM_420749	57 °C	495
Aggrecan core protein (AGR1)	Sense Antisense	CAA TGC AGA GTA CAG AGA (276–294) TCT GTC TCA CGG ACA CCG (688–704)	XM_001232949	54 °C	430
Collagen II (COL2A1)	Sense Antisense	GGA CCC AAA GGA CAG ACG G (1191–1210) TCG CCA GGA GCA CCA GTT (1573–1591)	NM_204426	59 °C	401
Sox9	Sense Antisense	CCC CAA CGC CAT CTT CAA (713–731) CTG CTG ATG CCG TAG GTA (1075–1093)	NM_204281	54 °C	381
GAPDH	Sense Antisense	GAG AAC GGG AAA CTT GTC AT (238–258) GGC AGG TCA GGT CAA CAA (775–793)	NM_204305	54 °C	556

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 (see Table 1), 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 a 1.2% ethidium bromide-containing agarose gel. Optical density of PCR product signals was determined by using ImageJ (Image Processing and Analysis in Java) version 1.46 freeware (http://rsbweb.nih.gov/ij/).

2.8. SDS–PAGE and Western blot analysis

Total cell lysates of HDC for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) were prepared as described previously [26]. 50 μg of protein was separated by 7.5% SDS–PAGE gel for immunological detection of key proteins for Ca^{2+} influx or release functions (*i.e.* pan α_1 subunit of voltage-gated Ca^{2+} channels and STIM1), as well as protein expression and phosphorylation status of the chondrogenic master transcription factor Sox9. Proteins were transferred electrophoretically to nitrocellulose membranes. After blocking in 5% non-fat dry milk in PBS, membranes were incubated with primary antibodies overnight at 4 °C as follows: rabbit polyclonal anti-Ca_V pan α_1 subunit in 1:200, epitope: intracellular C-terminus (Alomone Labs, Jerusalem, Israel); and mouse monoclonal anti-STIM1 in 1:500, epitope: 25–139 in human that has a high similarity to the chicken sequence (BD Biosciences, Franklin Lakes, NJ, USA); rabbit polyclonal anti-Sox9 antibody (Abcam, Cambridge, UK) in 1:600; rabbit polyclonal anti-P-Sox9 antibody (Sigma) in 1:800; rabbit polyclonal anti-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit polyclonal anti-GAPDH antibody (Abcam). After washing for 30 min in PBST, membranes were incubated with the HRP-conjugated secondary antibody, anti-rabbit IgG (Bio-Rad) in 1:1500 dilution. Membranes were developed by enhanced chemiluminescence reaction (Millipore, Billerica, MA, USA) according to the instructions of the manufacturer. Optical density of signals was measured by using ImageJ 1.46.

2.9. Statistical analysis

All data are representative of at least three independent experiments. Averages are expressed as mean ± SEM (standard error of the mean; *n*, number of cells measured). Statistical analysis was performed by using Student's *t*-test. Threshold for statistically significant differences as compared to respective control cultures was set at $^*P < 0.05$.

3. Results

3.1. Rapid spontaneous Ca^{2+} oscillations are detectable in differentiating chondrocytes

To investigate spontaneous repetitive transient increases in cytosolic Ca^{2+} concentration in individual cells of primary chondrifying micromass cultures derived from embryonic limb buds, Fluo-4 fluorescent Ca^{2+} imaging technique was applied on days 1 and 2 using LIVE confocal microscopy. As these cultures are heterogeneous by nature in terms of cellular composition (epithelial cells and muscle progenitors with distinct morphology can also be found in relatively small numbers along with osteochondroprogenitor cells; see [21]), only cells with round morphology were included in this study, while others were disregarded. Series of X–Y images

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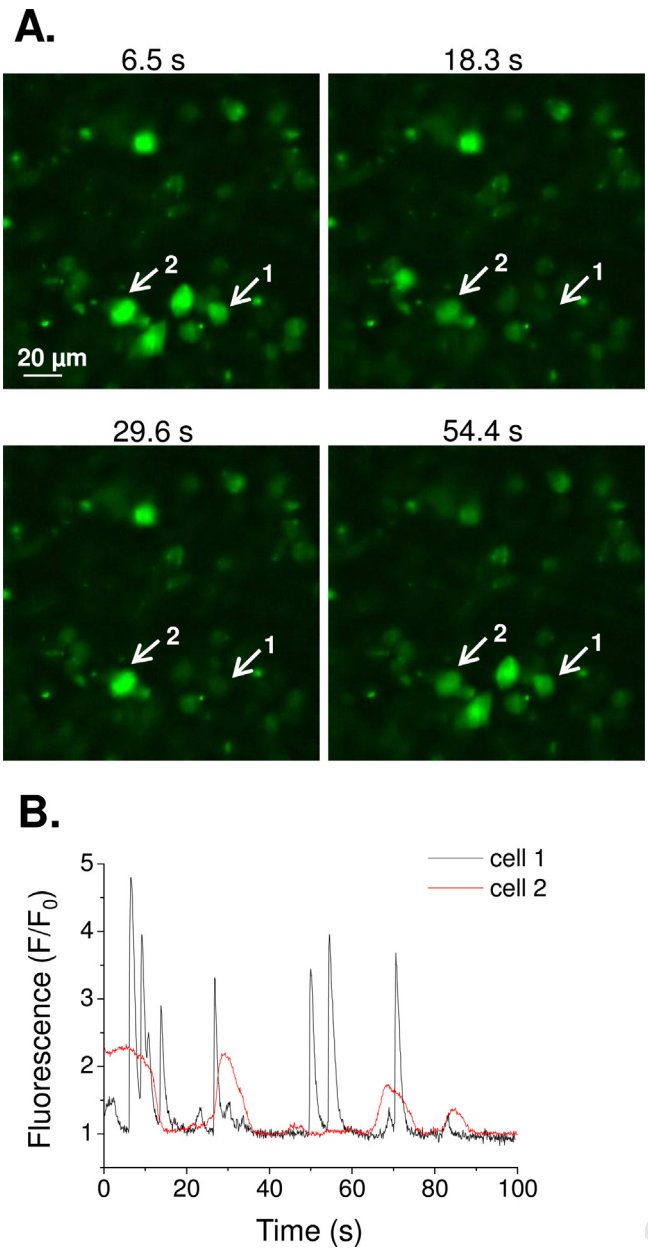

Fig. 1. Spontaneous Ca^{2+} oscillations in cells of HDC on day 2 of culturing. Prior to measurements, cells were loaded with Fluo-4-AM for 30 min Ca^{2+} oscillations were observed without agonist stimulation in Tyrode's solution containing 1.8 mM Ca^{2+} at room temperature. (A) Series of X–Y images were recorded from random visual fields of chondrifying cultures with Zeiss LIVE 5 Laser Scanning Confocal Microscope. These four representative frames were acquired at 6.5, 18.3, 29.6 and 54.4 s during measurements. Arrows indicate differentiating chondrocytes with repetitive intracellular Ca^{2+} oscillations. (B) Time course of fluorescence intensities of the cells marked with arrows in panel (A). Fluo-4 fluorescence intensity values normalised to baseline fluorescence (F/F_0) are plotted *vs.* time. Wide ranges of frequency and amplitude of oscillating cells were observed.

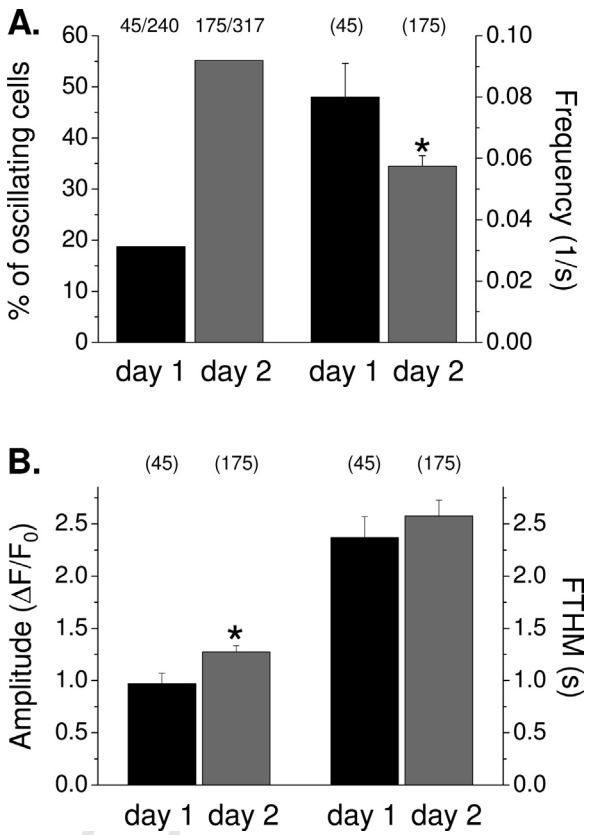

Fig. 2. Pooled data of Ca^{2+} oscillations gathered from series of X–Y images acquired from random visual fields of Fluo-4 loaded HDC on culturing days 1 and 2 with Zeiss LIVE 5 Laser Scanning Confocal Microscope. (A) Ratio of oscillating cells and frequency of repetitive Ca^{2+} transients on days 1 and 2 of culturing. Numbers above bars indicate the number of oscillating cells compared to all cells recorded. (B) Amplitude and full time at half maximum (FTHM) of Ca^{2+} oscillations in differentiating cells of HDC on culturing days 1 and 2. For both panels (A) and (B), while calculating the parameters of Ca^{2+} oscillations, only oscillating cells with round, chondroblast-like morphology were considered. Measurements were carried out on cultures from 4 independent experiments. Data represent mean ± standard error of the mean (SEM). Numbers in parentheses above bars indicate the number of cells measured. Asterisks (*) mark significant differences ($^*P < 0.05$) between parameters of oscillating cells in 1- and 2-day-old HDC.

were recorded from random visual fields of Fluo-4-loaded chondrifying cultures on days 1 and 2 of culturing. Ca^{2+} oscillations were recorded from cells bathed in Tyrode's solution containing 1.8 mM Ca^{2+} without agonist stimulation at room temperature. Four representative X–Y images recording Fluo-4-loaded cells in a 2-day-old culture taken at 6.5, 18.3, 29.6 and 54.4 s during measurements are shown in Fig. 1A. Wide ranges of frequency and amplitude of oscillating cells were observed (Fig. 1B; see Supplementary Video). On culturing day 1, 45 of 240 cells investigated (19%) exhibited spontaneous Ca^{2+} oscillations; whereas on day 2, the proportion of cells to show transient increases in cytosolic Ca^{2+} concentration was substantially higher (175 of 317 cells; 55%) (Fig. 2A). Not only the proportion of oscillating cells, but also their parameters exhibited substantial changes during the course of differentiation (Fig. 2A and B). While the frequency of oscillations were found to be significantly smaller on day 2 (0.06 ± 0.003 Hz; $n = 175$) *vs.* day 1 (0.08 ± 0.01 Hz; $n = 45$; $P = 0.01$) (Fig. 2A), the average amplitude of transient increases in cytosolic Ca^{2+} concentration-related fluorescence ratio significantly increased from day 1 (expressed as $\Delta F/F_0$: 0.97 ± 0.11; $n = 45$) to culturing day 2 (1.27 ± 0.06; $n = 175$; $P = 0.03$) (Fig. 2B). The third variable that was used to describe the duration of individual spontaneous transients (full time at half maximum; FTHM) did not prove to be statistically different on the two culturing days investigated (2.37 ± 0.29 s; $n = 45$ on day 1 *vs.* 2.57 ± 0.15 s; $n = 175$ on day 2; $P = 0.55$) (Fig. 2B).

3.2. Altered extracellular ionic milieu and Ca^{2+} entry blockers modify the appearance and quantitative parameters of rapid spontaneous Ca^{2+} oscillations in a time-dependent manner

Parameters of spontaneous Ca^{2+} oscillations were examined on day 2 of culturing by recording series of X–Y images. In each culture, a random visual field with oscillating cells was set, and frames

were acquired in control conditions (normal Tyrode's solution; containing 1.8 mM Ca^{2+}) at the 0 time point, and then in the presence of test solutions (either 10 μM nifedipine; or 500 μM $LaCl_3$ and 1 μM YM-58483); 1, 3 and 5 min after changing the bath to the test solutions. Control measurements were performed on random visual fields in cultures at 0-, 1-, 3- and 5-min time points in normal Tyrode's solution. It should be noted that all three parameters of Ca^{2+} oscillations investigated in this study (*i.e.* ratio of oscillating cells; amplitude and frequency of oscillations) followed a steady decline in the 5-min time frame even in control conditions; therefore, all parameters following an intervention were normalised to their respective control values (data not shown).

Application of the dihydropyridine L-type Ca^{2+} channel blocker nifedipine did not cause significant changes in the ratio of oscillating cells as compared to untreated control cells recorded at 3- and 5-min time points (80.93 ± 25.78% [$P = 0.56$]; and 91.73 ± 28.95 [$P = 0.84$], respectively; $n = 43$ for all time points; Fig. 3A), and no significant changes were observed in the amplitude either (87.58 ± 11.89% [$P = 0.5$]; 69.44 ± 10.26% [$P = 0.16$]; and 75.83 ± 6.26% [$P = 0.13$] at 1-, 3- and 5-min time points; $n = 25$, 16 and 11, respectively; Fig. 3B). At the same time, nifedipine decreased the frequency (53.97 ± 8.52% [$P = 0.01$]; and 64.46 ± 12.88% [$P = 0.15$] at 3- and 5-min time points; $n = 16$ and 11, respectively; Fig. 3C) of Ca^{2+} oscillations; this parameter was found to be significantly different from the control at the 3-min time point. These results indicate that nifedipine interfered with the frequency of oscillations, rather than the actual number of oscillating cells and the amplitude of the Ca^{2+} transients. In other words, treatment with nifedipine did not abolish repetitive Ca^{2+} transients, suggesting that VOCCs are not the primary, although important factors to mediate this phenomenon.

By contrast, administration of the SOCE blockers significantly decreased the ratio of oscillating cells at all three time points (11.59 ± 6.95% [$P = 0.0006$]; 23.05 ± 13.58% [$P = 0.008$]; and 0.0% [$P = 0.0$] at 1-, 3- and 5-min time points, respectively; $n = 29$ for all time points) compared to the control (Fig. 3A); in particular, no oscillating cells could be observed in random visual fields after 5 min. At the same time, amplitudes of repetitive Ca^{2+} transients were also reduced (45.35 ± 7.53% [$P = 0.24$]; and 58.33 ± 12.85% [$P = 0.34$] at 1- and 3-min time points; $n = 2$ and 3, respectively; Fig. 3B), and these blockers also decreased the frequency of oscillations at the 3-min time point (45.45 ± 22.72% [$P = 0.13$]; $n = 3$; Fig. 3C).

Since these results suggest that ER Ca^{2+} stores play a determining role in regulating repetitive Ca^{2+} transients in differentiating cells of HDC, we aimed to further analyse the effects of blockade of store-operated Ca^{2+} channels, as well as removal of free extracellular Ca^{2+} ions from the bath solution with a higher temporal resolution on line-scan diagrams. In these measurements, only round-shaped differentiating cells that exhibited prominent Ca^{2+} oscillations were included and other cells were disregarded. Line-scan images of oscillating cells were recorded on day 2 of culturing in normal Tyrode's solution (Fig. 4A). In the 46 cells examined, the frequency and the amplitude of Ca^{2+} oscillations were found to be 0.08 ± 0.007 Hz and 1.44 ± 0.15, respectively. Then, measurements were continued by changing the entire volume of the bath solution on the cultures to the test solutions. When $LaCl_3$ and YM-58483 were applied to oscillating cells ($n = 10$), Ca^{2+} oscillations were blocked, and a severe disturbance in cytosolic Ca^{2+} was observed: small-amplitude arrhythmic fluctuations in basal cytosolic Ca^{2+} concentration, rather than periodic Ca^{2+} transients, were recorded; therefore, the analysis of Ca^{2+} concentration-related changes in relative fluorescence intensities could not be performed (Fig. 4B). With the ER Ca^{2+} stores depleted—*i.e.* when the SERCA-blocker CPA was co-applied with $LaCl_3$ and YM-58483—Ca^{2+} oscillations were immediately eliminated ($n = 10$) and no changes in cytosolic

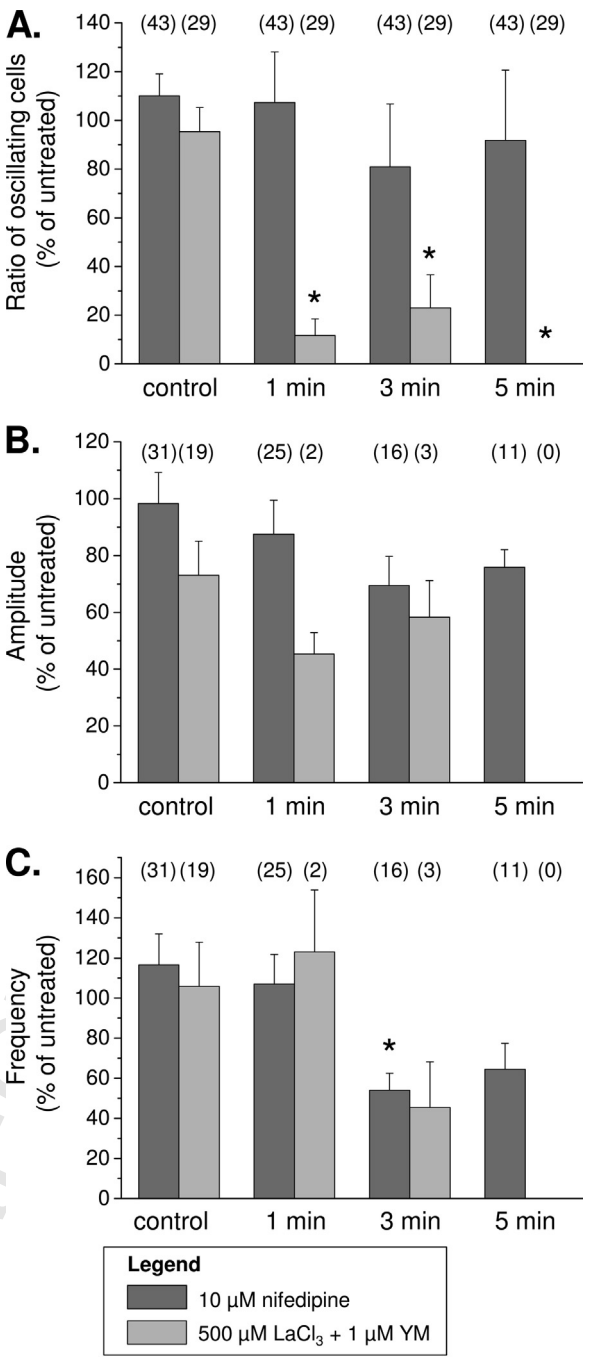

Fig. 3. Pooled data of Ca^{2+} oscillations obtained from series of X–Y images acquired from Fluo-4 loaded HDC in response to various treatments. Measurements were carried out with Zeiss LIVE 5 Laser Scanning Confocal Microscope. A total number of 500 images were recorded at each time point for each visual field; frame acquisition rate was 10 s^{-1}. (A) Percentage of oscillating cells before treatment (control), and 1, 3 or 5 min after the application of bath solution containing 10 μM nifedipine, or 500 μM $LaCl_3$ and 1 μM YM-58483. Values were normalised to the untreated cells measured at 0 min (control), and then at 1, 3 and 5 min. Numbers in parentheses above bars show the number of cells measured. (B) Amplitude of Ca^{2+} oscillations, normalised to values of untreated control cells. Numbers in parentheses above bars represent the number of oscillating cells measured. (C) Frequency of Ca^{2+} oscillations normalised to the control. Numbers in parentheses above bars show the number of cells measured. For panels (A)–(C), oscillating cells with round morphology in the same random visual field were recorded at all four time points. Differentiating cartilage colonies were only used for a single measurement series and then were discarded. Graphs represent pooled data of 3 independent experiments, measuring random visual fields of 5 colonies for each treatment. Asterisks (*) mark significant differences (*$P < 0.05$) between parameters of treated *vs.* control cells at respective time points.

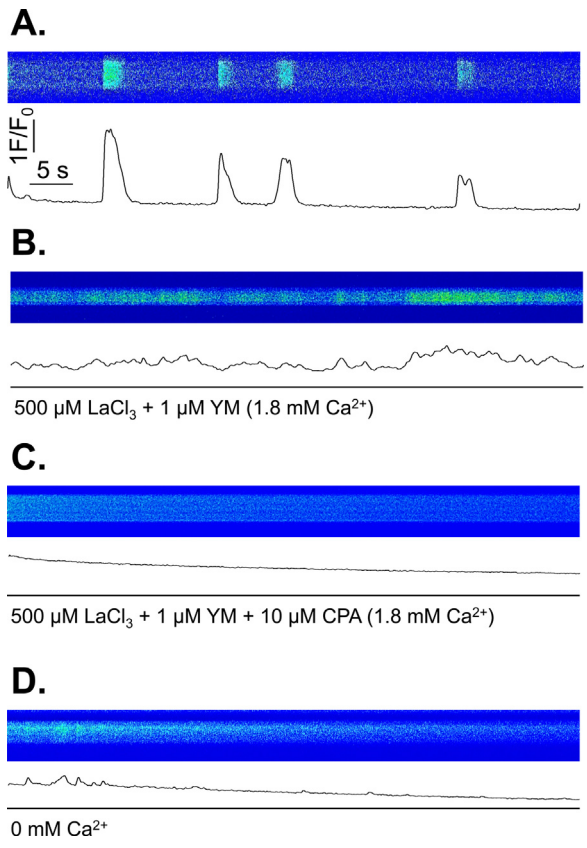

Fig. 4. Pharmacological modulation of spontaneous Ca^{2+} oscillations and effects of altered extracellular ionic milieu in Fluo-4 loaded differentiating chondrocytes of 2-day-old HDC. Representative confocal line-scan images and time courses of Fluo-4 fluorescence intensities are shown; horizontal and vertical calibrations are the same for all traces in panels (A)–(D). Horizontal lines under traces show the duration of treatments with pharmacons or altered extracellular ionic milieu. Acquisition of line-scan images started immediately after changing the bath solution on the cultures. Prior to that, normal functions were detected on each culture. (A) Spontaneous Ca^{2+} oscillations in normal ($[Ca^{2+}]_e = 1.8$ mM) Tyrode's solution. (B) After the non-selective cation channel-mediated Ca^{2+} entry blocker $LaCl_3$ (500 μM) and the store-operated Ca^{2+} entry and Ca^{2+} release-activated Ca^{2+} (CRAC) channel blocker YM-58483 (1 μM) were applied in normal ($[Ca^{2+}]_e = 1.8$ mM) Tyrode's, Ca^{2+} oscillations ceased, although irregular fluctuations in basal cytosolic Ca^{2+} concentration remained detectable. (C) When the SERCA-blocker CPA (10 μM) was co-administered with 500 μM $LaCl_3$ and 1 μM YM-58483 in 1.8 mM $[Ca^{2+}]_e$, Ca^{2+} oscillations were totally eliminated. (D) 3 min after changing the bath solution to Ca^{2+}-free Tyrode's, periodic oscillations could not be detected. Line-scan diagrams on panels (A)–(D) are representative data out of 4 independent experiments.

Ca^{2+} levels could be detected, even in the presence of external Ca^{2+} in the bath solution (Fig. 4C). When oscillating cells ($n = 10$) were bathed with Ca^{2+}-free Tyrode's solution, the amplitudes of oscillations gradually decreased, and only disappeared after several minutes. The representative line-scan diagram (Fig. 4D) shows lack of repetitive Ca^{2+} transients 3 min after changing the bath solution to Ca^{2+} free Tyrode's.

3.3. Differentiating chondrocytes express the α_1 subunit of VOCCs at both mRNA and protein levels

Since nifedipine was found to reduce the frequency of spontaneous Ca^{2+} oscillations; furthermore, application of 120 mM KCl evoked large Ca^{2+} transients in the same experimental model in our previous experiments [21], we can hypothesise that voltage-operated Ca^{2+} channels may be expressed and function on differentiating chondrocytes. Of the several different subunits that comprise functional VOCCs, the Ca^{2+} selective pore-forming α_1 subunit is the one that primarily determines the channel properties. Therefore, we first downloaded available sequence data for chicken α_1 subunit mRNAs, and carried out RT-PCR reactions with primer pairs specific for each type of VOCC designed by Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). Two ion channel subunit mRNA transcripts ($Ca_V1.2$ and $Ca_V1.3$) of L-type (or dihydropyridine-sensitive) Ca^{2+} channels were found to be expressed by chondrocytes, with $Ca_V1.2$ showing a constant expression level, while $Ca_V1.3$ exhibited a peak-like pattern with almost 5-fold stronger signals on days 2–4 of culturing (Fig. 5A). By contrast, the R-type $Ca_V2.3$ α_1 subunit mRNA showed a marked expression in chondroprogenitor mesenchymal cells, and gradually disappeared from differentiated chondroblasts and chondrocytes. The three T-type ($Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$) ion channel subunits, interestingly, followed a very similar mRNA expression profile to what has been observed in case of L-type channels; $Ca_V3.1$ and $Ca_V3.2$ followed a constant expression (with the latter exhibiting signs of downregulation in mature chondrocytes), whereas $Ca_V3.3$ also showed stronger signals during differentiation of chondroprogenitor cells on days 2–4 (Fig. 5A). Having confirmed mRNA expressions of various VOCCs, we also wanted to check the presence of α_1 subunits at the protein level. By using a polyclonal antibody raised against Ca_V α_1 subunits, immunoreactive bands were detected at the expected molecular weight (approx. 130 kDa). The protein showed strong expressions in total cell lysates of HDC throughout the entire culturing period (Fig. 5B). These findings, together with data obtained from Ca^{2+} imaging experiments, demonstrate the functional expression of various VOCCs in differentiating chondrogenic cells.

Next, we also undertook to characterise molecules that enable Ca^{2+} entry following store depletion (also known as Ca^{2+} release-activated Ca^{2+} or CRAC channels). Although Orai1 and STIM1, essential mediators of CRAC channel function, have been described in 2005 and 2006, respectively [27], their expression and function have not been investigated in chondrocytes. To this end, by designing specific primers, we were able to demonstrate the constant mRNA expression of STIM1, STIM2 and Orai1 throughout the entire culturing period (Fig. 5B), probably reflecting on their essential role in Ca^{2+} homeostasis. Furthermore, we also demonstrated the presence of STIM1 protein in total cell lysates of HDC on all culturing days, with a constant expression pattern (Fig. 5B). The identity of the upper immunogenic band at ~90 kDa is unknown; it can well be a glycosylated form or a splice variant of STIM1. Noteworthy that two STIM1 proteins of different size were found to be expressed in murine tissues: besides the well-known STIM1 isoform, a new 115 kDa STIM1 referred to as STIM1L has also been recently reported [28]. Owing to lack of commercially available chicken-specific antibodies raised against Orai1, Western blot analysis of this protein could not be performed.

3.4. Modulation of either VOCC function or SOCE in differentiating chondrocytes detrimentally affects in vitro chondrogenesis

To assess the long-term effects of the dihydropyridine L-type Ca^{2+} channel blocker nifedipine (applied at 10 μM continuously from day 1, due to constant expression of α_1 subunits) and SOCE inhibition combined with ER Ca^{2+} store depletion (by 500 μM $LaCl_3$, 1 μM YM-58483 and 10 μM CPA administered on day 2 for 24 h) on cartilage matrix production *in vitro*, further experiments were performed. Continuous treatment with nifedipine significantly attenuated cartilage matrix production by culturing day 6 as revealed by metachromatic staining procedures (Fig. 6A). With ER Ca^{2+} stores depleted, blockade of SOCE for only 24 h on day 2 resulted in an equally prominent inhibitory effect (Fig. 6A), reflecting on the important roles of both pathways (*i.e.* Ca^{2+} entry across the plasma membrane *via* VOCCs and Ca^{2+} release from internal Ca^{2+} stores) in Ca^{2+} homeostasis of differentiating chondrocytes.

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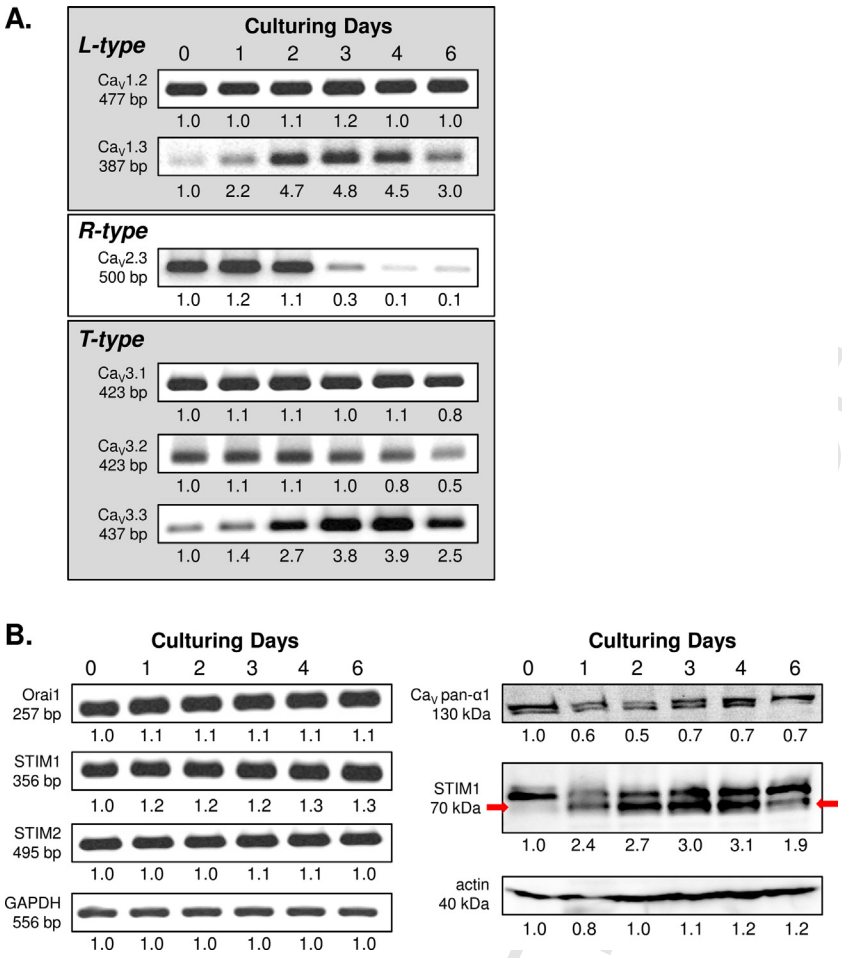

Fig. 5. Expression profiles of key molecules of Ca^{2+} influx and release on various days of culturing. (A) mRNA expression patterns of α_1 subunits of voltage-gated (L-, R-, and T-type) Ca^{2+} channels were detected by RT-PCR. (B) mRNA and protein-level expression profiles of molecules (STIM1/STIM2 and Orai1) that orchestrate the SOCE mechanism, and protein-level expression of the Ca^{2+} selective pore-forming α_1 subunit of voltage-gated Ca^{2+} channels on various days of culturing, detected with RT-PCR and Western blot analyses, respectively. For the STIM1 immunoblot, the lower band corresponds to the expected (70 kDa) molecular weight (marked by arrows). For PCR reactions shown in panels (A) and (B), GAPDH was used as a control (only shown in panel B). For Western blots, actin was used as a control. Data shown in panels (A) and (B) are representative out of 3 independent experiments. Numbers below bands represent integrated densities of signals determined by ImageJ freeware that were normalised to the value of day 0.

Noteworthy that combined treatments (with CPA, YM-58483 and $LaCl_3$) longer than 24 h completely abrogated chondrogenesis (data not shown). Day 2 was chosen to assess the effects of SOCE inhibition on the differentiation step of chondroprogenitor cells.

Next, we looked at whether these treatments interfered with the mRNA and protein expression of key genes of chondrogenesis; as mRNA expression of type 2 collagen (*COL2A1*) and aggrecan core protein (*AGR1*), as well as mRNA and protein expression and phosphorylation status of the key chondrogenic marker Sox9 were monitored on culturing day 3 by RT-PCR and Western blot analyses, respectively. Although continuous treatment with nifedipine did not interfere with the mRNA expression of these genes, Sox9 protein expression was found to be markedly reduced, without a detectable change in its phosphorylation level (Fig. 6B and C). By contrast, interference to ER Ca^{2+} store functions caused a strong downregulation of mRNAs for ECM components, although mRNA transcript levels of Sox9 were not altered (Fig. 6B). At the protein level, Sox9 protein expression and phosphorylation status followed the same changes as observed for treatments with nifedipine; Sox9 protein level was also found to be decreased, and the phosphorylation status was also only slightly modified following SOCE block (Fig. 6C).

Since in addition to modulating *in vitro* chondrogenesis at the molecular level, these treatments could have altered metabolic activity and/or the cell cycle of differentiating chondrocytes; therefore, mitochondrial activity and rate of proliferation were also determined on day 3 by MTT test and ^{3}H-thymidine incorporation assays, respectively. While neither nifedipine nor combined treatment to prevent Ca^{2+} re-uptake into depleted internal stores modulated cellular metabolic activity, both treatments almost completely abrogated cell proliferation (Fig. 6D). Consequently, the observed decrease in metachromatic matrix production can partially be attributed to the detected dramatic inhibition of cell proliferation.

3.5. SOCE blockers alter the parameters of store-operated Ca^{2+} entry induced by store depletion during fluorescent single cell Ca^{2+} measurements

Intracellular Ca^{2+} concentration measurements were performed in 2-day-old cultures loaded with Fura-2 to assess the parameters of Ca^{2+} transients triggered *via* SOCE in the absence (Fig. 7A) and in the presence of SOCE blockers (500 μM $LaCl_3$ and 1 μM YM-58483; Fig. 7B). At the beginning of recordings, internal Ca^{2+} stores of cells were emptied by pre-treatment with the SERCA-inhibitor CPA (10 μM) dissolved in Ca^{2+}-free Tyrode's (not shown). The changes in cytosolic Ca^{2+} concentration evoked by re-establishing the normal (1.8 mM) extracellular Ca^{2+} concentration were then recorded.

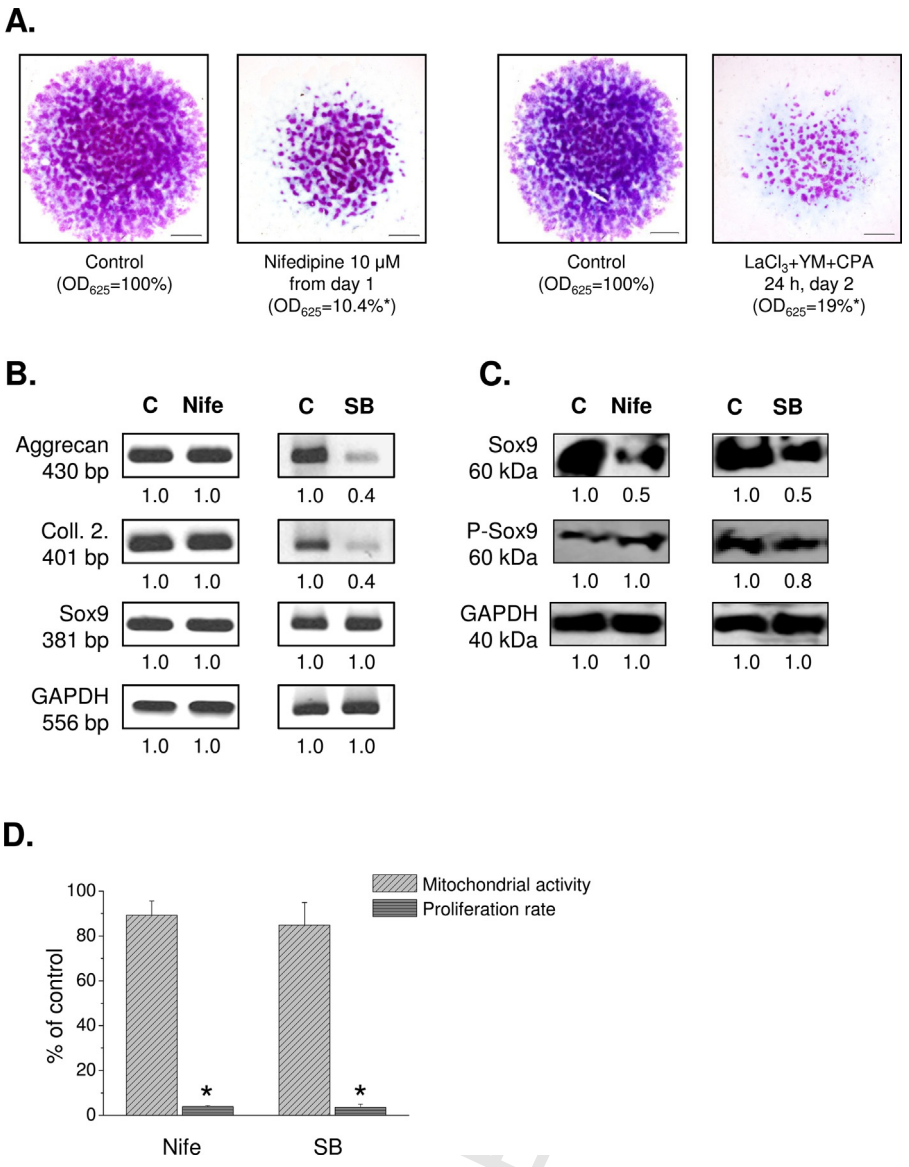

Fig. 6. Effects of the dihydropyridine L-type Ca^{2+} channel blocker nifedipine (applied continuously from day 1 at 10 μM) and SOCE inhibition combined with ER Ca^{2+} store depletion (by 500 μM $LaCl_3$, 1 μM YM-58483 and 10 μM CPA administered on day 2 for 24 h) on cartilage matrix production *in vitro*. (A) Metachromatic cartilage areas in 6-day-old high density colonies were visualised with DMMB dissolved in 3% acetic acid (pH 1.8). Metachromatic (purple) structures represent cartilaginous nodules formed by many cells and a cartilage matrix rich in polyanionic GAGs. Original magnification was 2×. Scale bar, 1 mm optical d (OD625) was determined in supernatants of 6-day-old cultures containing toluidine blue extracted with 8% HCl dissolved in absolute ethanol. (B) mRNA transcri key chondrogenic and ECM marker genes on day 3 analysed by RT-PCR. GAPDH was used as a control. (C) Protein expression and phosphorylation status of the master chondrogenic transcription factor Sox9 in 3-day-old cultures. GAPDH was used as a control. (D) Mitochondrial activity and rate of proliferation on day 3 determined by MTT test and ^{3}H-Thymidine incorporation assays, respectively. Statistically significant (*$P < 0.05$) differences in extinction (OD_{625}) of samples for TB and in rate of proliferation are marked by asterisks (*). Abbreviations used for panels (B)–(D): C, control; Nife, nifedipine; SB, SOCE block by $LaCl_3$, YM-58483 and CPA. Representative data out of 3 independent experiments are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Compared to untreated control cells, in the presence of the SOCE blockers both the amplitude (114.4 ± 15.6; $n = 16$ *vs.* 58.4 ± 5.6; $n = 10$; $P = 0.01$; Fig. 7C) and the maximal rate of rise (3.9 ± 0.5; $n = 16$ *vs.* 1.5 ± 0.2; $n = 10$; $P = 0.001$; Fig. 7D) of SOCE were significantly decreased.

4. Discussion

4.1. Ca^{2+} homeostasis in differentiating chondrocytes

It is generally accepted that Ca^{2+} is the most versatile second messenger. There is accumulating evidence that Ca^{2+} signalling pathways are key mediators of cellular events involved in differentiation processes also in non-excitable cells including MSCs and chondrocytes. A sustained rise in cytosolic Ca^{2+} concentration induces differentiation of MSCs: increased intracellular Ca^{2+} was reported to exert a biphasic regulatory role in adipocyte differentiation, inhibiting the early stages while promoting the late stage of differentiation [29]. Differentiation of mesenchymal cells into chondrocytes is also controlled by Ca^{2+} dependent pathways: high concentration of extracellular Ca^{2+} was found to promote chondrogenic differentiation in chicken HDC [30]. By contrast, extracellular Ca^{2+} was reported to modulate differentiation during skeletogenesis in chicken embryonic calvaria, where low concentrations enabled chondrogenesis [31]. The importance of Ca^{2+} influx *via* plasma membrane ion channels during chondrogenesis in mouse limb bud-derived HDC was confirmed by the fact that treatment with the L-type channel-specific blockers nifedipine and verapamil

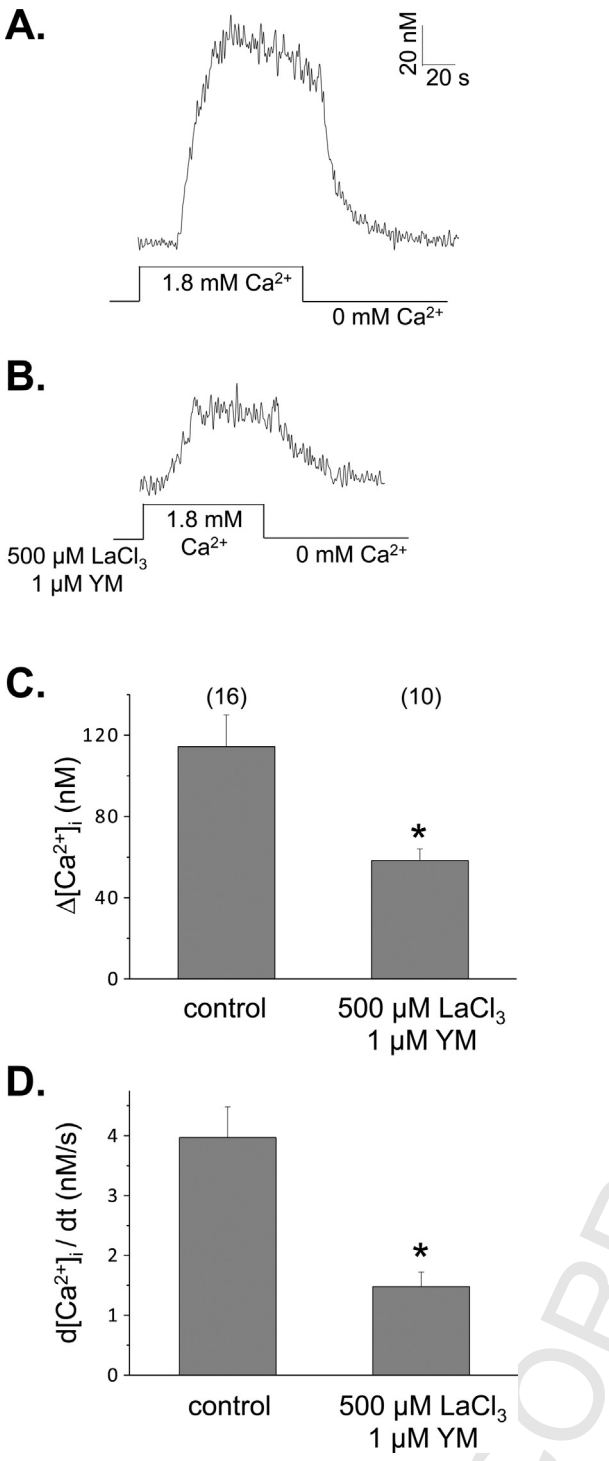

Fig. 7. Inhibition of store-operated Ca^{2+} entry (SOCE) in differentiating cells of Fura-2 loaded 2-day-old HDC during single-cell fluorescent Ca^{2+} imaging. Internal Ca^{2+} stores of cells were previously depleted by the application of 10 µM CPA in Ca^{2+}-free Tyrode's for 5 min, which caused a marked decrease in $[Ca^{2+}]_i$ following a prominent increase (not shown). (A) When $[Ca^{2+}]_e$ was changed back to normal (1.8 mM), a large transient increase in $[Ca^{2+}]_i$ was observed, demonstrating SOCE. (B) When SOCE was blocked by pre-treatment with 500 µM $LaCl_3$ and 1 µM YM-58483 for 5 min, Ca^{2+} entry considerably decreased. (C) Pooled data of amplitudes of transients evoked by Ca^{2+} entry. (D) Mean values of the maximal rate of rise of SOCE (calculated as $d[Ca^{2+}]_i/dt$) after the re-administration of external calcium. Numbers in parentheses indicate the number of cells measured. Asterisks (*) mark significant ($^*P < 0.05$) differences between control cultures and cells pre-treated with $LaCl_3$ and YM-58483. Traces in panels (A) and (B) are representative out of 16 and 11 records, respectively.

attenuated differentiation [32]. Consistent with the above findings, a sustained rise in cytosolic free Ca^{2+} concentration was found to coincide with the final commitment of differentiating chondroprogenitor cells in chicken HDC and proved to be a prerequisite to chondrogenic differentiation in these cultures [6].

4.1.1. VOCCs mediate Ca^{2+} influx during chondrogenesis

The above results concerning Ca^{2+} homeostasis and Ca^{2+} dependent signalling events during chondrogenesis suggest that chondrogenic differentiation partially depends on the availability of extracellular Ca^{2+} and that their influx *via* plasma membrane Ca^{2+} channels is indispensable. Indeed, many Ca^{2+} entry pathways have been identified in developing and mature chondrocytes by our laboratory and others, including P2X and P2Y purinergic receptors, *N*-methyl-D-aspartate receptor (NMDA) subunits, transient receptor potential (TRP) channels and arachidonate-regulated Ca^{2+}-sensitive (ARC) channels [22]. NMDA receptors and TRPV ion channels are of particular importance since they are key mediators of biomechanical signals and are thus significant components of chondrocyte mechanotransduction pathways [33,34]. Besides these ligand-gated Ca^{2+} entry pathways, the functional expression of voltage-operated Ca^{2+} channels (VOCCs) has also been reported during chondrogenesis. VOCCs are large complexes consisting of several subunits (α_1, $\alpha_2\delta$, β_{1-4}, and γ), of which the α_1 subunit determines their type, *i.e.* L- ($Ca_V1.1$, 1.2, 1.3, 1.4), P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), R- ($Ca_V2.3$) and T-type ($Ca_V3.1$, 3.2, 3.3) Ca^{2+} channels [35]. Voltage-gated Ca^{2+} channels have long been known to be expressed by articular chondrocytes and contribute to elevation of cytosolic Ca^{2+} concentration *via* signalling through insulin-like growth factor-1 [36]. VOCCs were reported to co-localise with beta-1 integrin, Na^+/K^+-ATPase and epithelial sodium channels in mechanoreceptor complexes in developing chondrocytes [37]; furthermore, expressions of $Ca_V1.2$ and $Ca_V3.2$ channel subunits were detected in chondrocytes derived from developing mouse embryos by immunohistochemical staining procedures and in the murine chondrogenic cell line ATDC5 [38]. Xu et al. recently demonstrated that Ca^{2+} influx *via* VOCCs is also necessary for signalling pathways in stimulated articular chondrocytes [39].

However, a detailed molecular characterisation of various VOCCs expressed in differentiating chondrogenic cells was lacking. Preliminary findings of our laboratory have implicated the involvement of various voltage-gated Ca^{2+} channels during *in vitro* chondrogenesis in chicken HDC as exposure to high concentrations of extracellular K^+ evoked large Ca^{2+} transients [21]. Therefore, we undertook to provide a detailed analysis of mRNA expression profiles of α_1 subunits and found mRNA transcripts of two L-type ($Ca_V1.2$ and $Ca_V1.3$), an R-type ($Ca_V2.3$) and all three T-type ($Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$) Ca^{2+} channels. Furthermore, we also confirmed the presence of α_1 subunit proteins in total cell lysates throughout the entire culturing period, suggesting that VOCCs may play a role during the differentiation of chondroprogenitor cells to chondroblasts and mature chondrocytes. The presence of the α_1 subunit in chondrocytes was first confirmed by Shakibaei and Mobasheri in 2003 [37]. Since then, Ca^{2+} channel expression in chondrocytes has been implied also by others and suggested that they are involved in mechanotransduction pathways [40]. Furthermore, both differentiating hMSCs and limb bud-derived chondroprogenitor mesenchymal cells were found to express L- and T-type Ca^{2+} channels, which probably reflects on the role of these specific channels in controlling Ca^{2+} homeostasis during differentiation. Although the molecular and functional characterisation of VOCCs during osteogenic differentiation of hMSCs has been published [41], this is the first study to provide a comprehensive analysis of VOCC expression during *in vitro* chondrogenesis.

As far as the function of VOCCs during regulation of chondrogenesis is concerned, when the L-type channel specific inhibitor

nifedipine was administered to differentiating HDC from day 1, a markedly reduced cartilage ECM production was detected by the end of the 6-day-long culturing period. Consistent with this, Sox9 protein expression was considerably attenuated. These findings are in a good correlation with results gained earlier on mouse embryonic limb bud mesenchymal cells, where chondrogenesis was inhibited by continuous application of nifedipine [32]. By contrast, nifedipine did not interfere with osteogenic differentiation of hMSCs [41]. However, this contradiction may be resolved if one hypothesises that the Ca^{2+}-dependent molecular machinery that regulates osteo- and chondrogenesis may be different; moreover, differentiation steps of adult bone marrow MSCs (which are multipotent and give rise to many cell types) and embryonic chondroprogenitor mesenchymal cells (that are already committed towards the chondrogenic lineage) may also utilise different regulatory pathways.

VOCCs (in particular, L- and T-type) have long been known to regulate cell proliferation [42]. The concept that the control of the cell cycle is at least partially Ca^{2+} dependent has been proposed some 40 years ago but this field is still controversial. Both Ca^{2+} influx from extracellular space and Ca^{2+} release from internal stores were reported to govern cell proliferation; the dependence of progression through cell cycle on Ca^{2+} influx may rely on ER Ca^{2+} contents as it substantially varies from one cell type to another [43]. Of the plasma membrane Ca^{2+} ion channels, VOCCs (especially T-type channels) may play a determining role in cell cycle regulation in non-excitable cells by allowing for a constitutive Ca^{2+} influx that is very sensitive even to modest changes in resting membrane potential (RMP) owing to their unique low voltage-dependent activation/inactivation and slow deactivation properties [43]. Noteworthy that cells of chondrifying HDC were found to express all three T-type channel mRNAs throughout the entire culturing period in this study, probably reflecting on the important role of these VOCCs in Ca^{2+} homeostasis during chondrogenesis. Since *in vitro* chondrogenesis requires high cellular density, block of voltage-operated Ca^{2+} channels could have interfered with chondrogenic differentiation of HDC by inhibiting cellular proliferation. Indeed, application of the L-type channel blocker nifedipine exerted antiproliferative effects in differentiating chondrocytes as it was revealed by monitoring the rate of DNA synthesis. Therefore VOCCs, besides mediating long-lasting Ca^{2+} influx and maintaining basal cytosolic Ca^{2+} levels, may be important regulators of cell division required for condensation of mesenchymal cells prior to chondrogenic differentiation in response to changes of RMP brought about by *e.g.* voltage-dependent K^+ channels (such as $K_V1.1$ and $K_V1.3$) also expressed by cells of HDC [21].

4.1.2. STIM1/STIM2 and Orai1 mediate store-operated Ca^{2+} entry (SOCE) in differentiating chondrocytes

In addition to Ca^{2+} entry across the plasma membrane mediated by either voltage or ligand gated ion channels, a further source of transient increases of cytosolic Ca^{2+} concentration is Ca^{2+} release from the ER Ca^{2+} stores *via* Ca^{2+} permeable IP_3R or RyR channels [44]. Ca^{2+} content of internal pools primarily depends on Ca^{2+} uptake through SERCA and/or Na^+–Ca^{2+} exchanger (NCX), buffering capacity of ER Ca^{2+} binding proteins, and Ca^{2+} release. Non-excitable cells preferentially utilise pathways that trigger the activation of IP_3Rs, rather than Ca^{2+} release *via* RyR [45]. In fact, administration of caffeine, a strong agonist that activates RyR had no effect either in mature [46] or in differentiating chondrocytes [6], which is consistent with what has been observed in human and murine mesenchymal and embryonic stem cells [5,47]. Furthermore, while IP_3R mRNA and protein expression was demonstrated in differentiating chondrocytes (and also in human MSCs), no mRNA transcripts for RyR were detectable either in MSCs or in cells of HDC [5,6]. Given that the ER Ca^{2+} stores in non-excitable cells including chondrocytes are relatively small, signalling pathways that generate the lipid metabolite IP_3, which triggers Ca^{2+} release *via* IP_3Rs may lead to store depletion. This, in turn, activates Ca^{2+} entry across the plasma membrane, a process termed store-operated Ca^{2+} entry (SOCE), accomplished by the activation of CRAC channels to refill the ER stores [44]. SOCE has been shown to contribute to maintenance of Ca^{2+} homeostasis in many non-excitable cells including both rat primary chondrocytes [48] and in the human chondrosarcoma cell line OUMS-27 [49]; furthermore, we also demonstrated earlier that upon store depletion brought about by preventing Ca^{2+} re-uptake into the ER using the SERCA pump inhibitor CPA, differentiating chondrocytes exhibited large transient rise in cytosolic Ca^{2+} concentration when extracellular Ca^{2+} became available [6]. However, CRAC channels and molecules involved in SOCE in either mature or developing chondrocytes have not been extensively characterised.

SOCE is a complex series of events initiated by oligomerisation of the Ca^{2+} sensor stromal interaction molecule (STIM1) and STIM2 in the ER membrane triggered by a decrease in ER Ca^{2+} content below a certain threshold. Oligomerisation of STIM1/STIM2 is followed by redistribution into distinct puncta in the ER membrane that are located in close proximity to the plasma membrane, where STIM directly interacts with Orai1 dimers to form tetramers, thus giving rise to the functional CRAC channel [27].

Chondrocytes have long been known to possess releasable ER Ca^{2+} stores [50]; furthermore, as mentioned above, our laboratory has also demonstrated releasable Ca^{2+} stores in cells of differentiating cartilage [6]. However, the detailed characterisation of Ca^{2+} transients induced by SOCE was lacking. This is the first study to provide direct evidence on the functional expression of the molecules that mediate SOCE in differentiating chondrocytes. mRNA transcripts of Orai1, as well as STIM1 and STIM2 were found to be expressed in cells of HDC at constant levels throughout the entire culturing period. Furthermore, STIM1 expression at the protein level has also been confirmed. STIM2 may also have important functions in developing chondrocytes as it is activated by smaller decreases in ER Ca^{2+} concentrations than STIM1 and therefore it has been proposed to regulate basal cytosolic Ca^{2+} concentrations [51]. Owing to species limitations (lack of commercially available antisera raised against chicken Orai1) we were unable to demonstrate the presence of the Orai1 protein. Nevertheless, the fact that store-operated Ca^{2+} entry was recorded in cells of HDC during single cell fluorescent Ca^{2+} measurements provides indirect evidence on the protein expression of the pore-forming subunit of CRAC channels. Furthermore, SOCE mediated by interaction between STIM and Orai1 seems to be a very important contributor to normal Ca^{2+} homeostasis of differentiating chondrocytes as blockade of SOCE combined with ER Ca^{2+} store depletion by preventing re-uptake with continuous application of CPA almost completely abrogated *in vitro* chondrogenesis. Insufficient replenishment of ER Ca^{2+} stores and consequently disturbed Ca^{2+} homeostasis may modulate cartilage formation by detrimentally affecting cell division. Indeed, proliferation rate in cells with depleted internal Ca^{2+} stores was found to be almost completely attenuated, without severely affecting mitochondrial metabolic activity. These results further support the concept that the cell proliferation machinery in differentiating chondrocytes is very sensitive to changes in cytosolic Ca^{2+} levels, irrespective of the source of Ca^{2+} (either Ca^{2+} entry across the plasma membrane or Ca^{2+} release from internal stores); notwithstanding the fact that the relationship between ER Ca^{2+} contents and cell cycle regulation is controversial and seems to be cell type-specific as discussed by Capiod [43].

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4.2. Possible role of Ca^{2+} oscillations during chondrogenesis

Many studies have shown that the frequency of biological oscillations encode dynamical information to control varied cellular activities including gene activation, cell division, or proliferation. In particular, periodic Ca^{2+} oscillations are generally accepted to enhance efficiency and specificity of signalling pathways at lower levels of stimulation than a small, sustained increase in basal Ca^{2+} levels [52]. Therefore, Ca^{2+} oscillations are considered to be more suitable for cells that only receive low levels of stimulation and/or characterised by low receptor occupancies. It is therefore not surprising that periodic fluctuations in basal cytosolic Ca^{2+} concentration have been observed in many non-excitable cells including MSCs and chondrocytes.

The concept of an intracellular "pulsatile Ca^{2+} pacemaker" region that is capable of initiating and maintaining rhythmic Ca^{2+} oscillations in isolated articular chondrocytes was first proposed by D'Andrea and Vittur in 1995 [50]. Later on, others have also reported on spontaneous Ca^{2+} oscillations in sliced cartilage and primary chondrocyte cultures; furthermore, mechanically induced Ca^{2+} waves regulated by a paracrine purinergic signalling pathway have also been described [53]. This is particularly important as Ca^{2+} oscillations and consequent activation of NFAT that governs proliferation and differentiation were also found to be regulated by an ATP autocrine/paracrine loop in human MSCs [54]. Interestingly, however, in the murine chondrogenic teratocarcinoma cell line ATDC5, Ca^{2+} oscillations were found to drive ATP oscillations that play a critical role during prechondrogenic condensation and oscillatory secretion of ECM and cell adhesion molecules [55]. Furthermore, the same laboratory has proposed that these Ca^{2+}-driven ATP oscillations are initiated by extracellular ATP signalling *via* the ionotropic purinergic receptor $P2X_4$ [56]; the same receptor that was suggested by our laboratory to be a key player in mediating Ca^{2+} influx in differentiating chondrocytes [20]. Nevertheless, a reciprocal regulatory loop between ATP and Ca^{2+} oscillations in differentiating MSCs and chondrocytes can be hypothesised that may drive differentiation and/or proliferation of these cell types.

As far as temporal aspects of Ca^{2+} oscillations in non-excitable cells are concerned, huge variations exist. For MSCs, the average period of cyclic Ca^{2+} transients was 5.7 ± 3.0 min [5] with large individual variability. Cells in primary cultures of human osteoblasts were also reported to exhibit spontaneous oscillations with a frequency of 0.25–0.6 min^{-1} [57], which correlates well with the range observed in MSCs. In chondrocytes, however, oscillations exhibited greater variability. In a slice of mature rabbit articular cartilage, individual chondrocytes exhibited spontaneous long-lasting (up to 30 s) Ca^{2+} transients within a 120-s-long observation period [53]. Cytosolic Ca^{2+} (and subsequently ATP) in the chondrogenic ATDC5 cell line were found to oscillate with a period of 1 transient in approx. every 6 h [55]. By contrast, our measurements performed on differentiating cells of chicken micromass cultures revealed spontaneous Ca^{2+} oscillations at a significantly higher frequency. In 45 of 240 cells (19%) and in 175 of 317 cells (55%) examined during this study on culturing days 1 and 2, respectively, Fluo-4-loaded cells in HDC exhibited cyclic Ca^{2+} transients at a rate of 0.08 ± 0.01 Hz on day 1, which decreased to 0.06 ± 0.003 Hz by culturing day 2. In a recent study performed by Nguyen et al., Fluo-4 loaded bovine articular chondrocytes were found to exhibit oscillations at a comparably high frequency to cells of HDC; furthermore, these oscillations were also dependent on both extracellular and intracellular Ca^{2+} sources, as well as relied on the function of voltage-dependent and voltage-independent Ca^{2+} channels [58]. However, in this work, oscillations were induced by basic calcium phosphate (BCP) crystals to mimic microcrystalline stress, a phenomenon involved in OA pathogenesis; moreover, the authors concluded that BCP crystal-induced Ca^{2+} oscillations were deleterious as they were involved in proteoglycan degradation. By contrast, spontaneous Ca^{2+} transients in limb bud-derived chondrifying cultures seemed to be inherent features of differentiating cells of HDC.

4.3. Involvement of Ca^{2+} entry and release processes in Ca^{2+} oscillations

Although we have investigated the source of Ca^{2+} flux that generates transient increases in cytosolic Ca^{2+} in a previous study [21], we now performed a more thorough analysis. We found that lack of free Ca^{2+} in the bath solution abrogated oscillations after a few minutes; this implies the dependence of the phenomenon on Ca^{2+} entry across the plasma membrane, and at the same time provides evidence that internal stores also contribute to the maintenance of oscillations, but only to a limited extent, probably owing to their relatively low Ca^{2+} contents. This finding is in accordance with what has been described in hMSCs, since this group has also concluded that Ca^{2+} entry was required to sustain Ca^{2+} oscillations [5]. Similar results were observed also in differentiating human osteoblast-like cells [57]. To identify Ca^{2+} channels that maintain Ca^{2+} entry during periodic transients, we examined whether VOCCs that proved to be important regulators of sustained Ca^{2+} transport across the plasma membrane were involved in this process. When the L-type Ca^{2+} channel blocker nifedipine was applied during fluorescent Ca^{2+} imaging, it slightly reduced the ratio of oscillating cells, as well as parameters (amplitude and frequency) of Ca^{2+} oscillations compared to untreated controls. These results indicate that Ca^{2+} influx *via* VOCCs indeed plays a role in maintaining Ca^{2+} oscillations, but their contribution is only secondary. By contrast, application of nifedipine on osteoblasts had no effect on Ca^{2+} oscillations [57].

Next, we looked at the role of internal Ca^{2+} stores. When $LaCl_3$ and YM-58483, blockers that inhibit all channel-mediated Ca^{2+} entry and CRAC channels, respectively, were applied to oscillating cells, a severe disturbance in cytosolic Ca^{2+} was observed: small-amplitude fluctuations, rather than periodic Ca^{2+} transients, were recorded. Furthermore, with the ER Ca^{2+} stores depleted—*i.e.* when the SERCA-blocker CPA was co-applied with $LaCl_3$ and YM-58483—no changes in cytosolic Ca^{2+} levels could be detected, even in the presence of external Ca^{2+} in the bath solution. Therefore, Ca^{2+} cycling between intracellular pools and the cytosol seems to be a determining factor in the generation of spontaneous Ca^{2+} transients as blockade of SOCE combined with store depletion completely abrogated oscillations. These results are in a complete agreement with what has been observed in osteoblasts where oscillations could also be blocked by store depletion induced with thapsigargin [57]. Since Kawano et al. have also reported on the dependence of spontaneous Ca^{2+} oscillations on internal Ca^{2+} pools in hMSCs [5], the determining role of ER Ca^{2+} stores and processes of store replenishment (SOCE; including STIM and Orai isoforms) in the generation/maintenance of Ca^{2+} oscillations seems to be a general phenomenon in differentiating mesenchymal cells.

4.4. Molecular decoders of Ca^{2+} oscillations

It is widely accepted that Ca^{2+} acts *via* activation of pathways that involve Ca^{2+}-dependent protein kinases (classic PKCs, *e.g.* PKCalpha) and phosphoprotein phosphatases (calcineurin); these in turn trigger various transcription factors such as NFAT, NF-κB, JNK1, MEF2 and CREB, depending on the frequency and amplitude of the Ca^{2+} transients. In lymphocytes and MSCs, Ca^{2+} oscillations were reported to trigger the calcineurin-dependent dephosphorylation and consequent nuclear translocation of NFAT [54]. The importance of the calcineurin–NFAT4 pathway has been demonstrated in chondrogenesis [59,60]. These data indicate that NFAT4 activation induced by high-frequency Ca^{2+} oscillations may play

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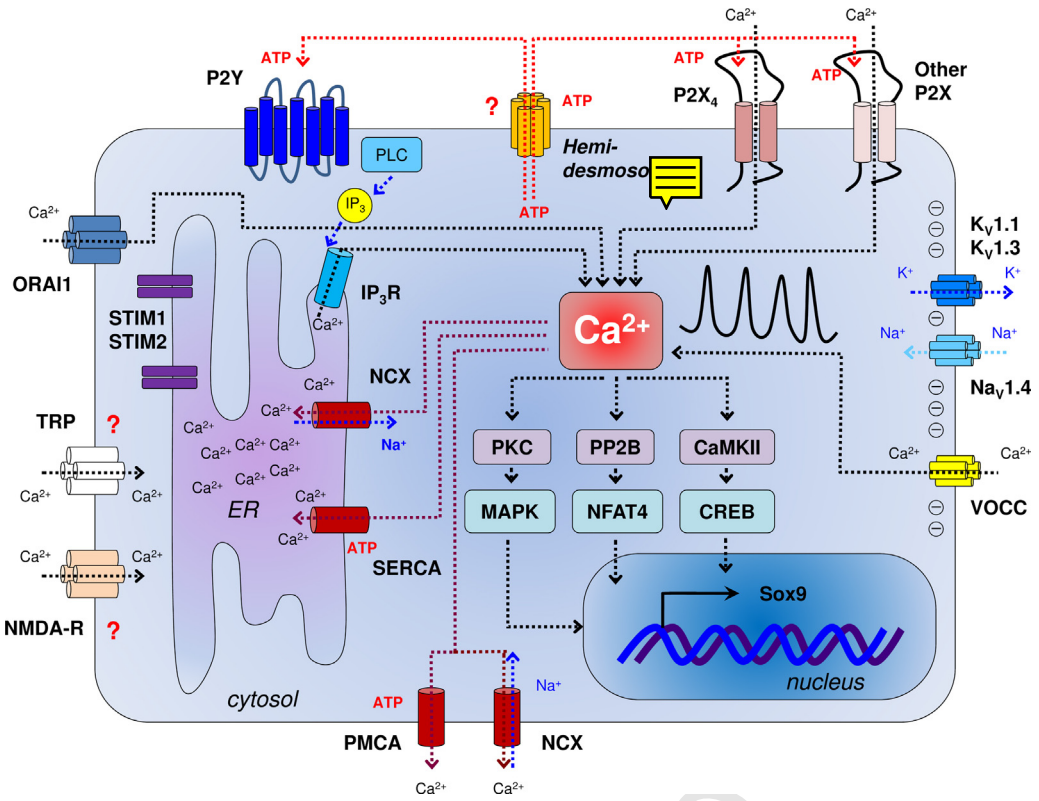

Fig. 8. Refined model showing known components of Ca^{2+} homeostasis and signalling pathways that modulate Ca^{2+} oscillations in developing chondrocytes. Ca^{2+} can enter the cell *via* voltage-operated Ca^{2+} channels (VOCCs), $P2X_4$ purinergic receptors, NMDA receptors or TRP channels; changes in resting membrane potential are mediated by voltage-gated K^+ channels. ATP is secreted to the extracellular space *via* putative connexin 43 hemichannels. Activation of G-protein coupled P2Y purinergic receptors cause Ca^{2+}-release from the endoplasmic reticulum (ER) *via* inositol-1,4,5-trisphosphate receptors (IP_3R). Depletion of the Ca^{2+} stores cause aggregation of stromal interaction molecules (STIM1/STIM2), which are the Ca^{2+} sensors of the ER and trigger the opening of the store-operated Orai1 channels. Reuptake of Ca^{2+} to the ER is mediated by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the Na^+–Ca^{2+} exchanger (NCX). See text for details.
The concept of the image was adopted from [44].

important roles in regulating proliferation and differentiation during chondrogenesis.

Cyclic rise in basal cytosolic Ca^{2+} concentration may also trigger the activation of Ca^{2+}–calmodulin dependent protein kinase II (CaMKII) that has been reported to act as a decoder of Ca^{2+} oscillations [61], owing to its complex regulation and progressive activation. CaMKII mediates many cellular functions in response to elevated Ca^{2+} in various cells and tissues, including regulation of ion channels, cytoskeleton, gene transcription and proliferation. Expression of various CaMKII enzymes has been confirmed in human articular chondrocytes at both mRNA and protein levels [62]; furthermore, we have also detected mRNA transcripts of CaMKII isoforms alpha, beta, gamma and delta in differentiating cells in HDC with variable expression patterns (Supplementary Fig. 1). CaMKII is especially suitable to decode high-frequency Ca^{2+} oscillations as its activation is sensitive to periodic changes in the range of 0.1–10 s [63]. For it is exactly at this range that differentiating cells in our experimental model exhibit spontaneous Ca^{2+} oscillations, it can be hypothesised that this pathway may mediate oscillation-linked activation of transcription factors to promote chondrogenic differentiation and matrix synthesis in primary chicken micromass cultures. However, further studies are needed to unequivocally confirm the involvement of CaMKII in decoding cytosolic Ca^{2+} oscillations in differentiating chondrocytes.

4.5. Refined model of Ca^{2+} homeostasis in differentiating chondrocytes with respect to Ca^{2+} oscillations

Based on results obtained in the present work and earlier data of our research group, as well as studies performed by others in this field, the following model is proposed to describe the generation and maintenance of spontaneous Ca^{2+} oscillations in differentiating chondrocytes (Fig. 8). Both Ca^{2+} entry across the plasma membrane and Ca^{2+} release from internal stores are required for the phenomenon, since oscillations disappeared when cultures were bathed in Ca^{2+}-free extracellular solution, and also when internal Ca^{2+} stores were depleted. Therefore, at least three distinct mechanisms may be responsible for generation of Ca^{2+} oscillations.

First, an autocrine–paracrine purinergic regulatory loop during *in vitro* chondrogenesis first proposed in 2009 by our group [20] can be considered, similarly to what has been described in mature chondrocytes [53] or in MSCs [54]. We and others have reported that ATP is secreted by differentiating chondrocytes [20], possibly through connexin 43 hemichannels [64]; extracellular nucleotides may act on the two large superfamilies of P2 purinergic receptors: the ATP-gated ion channel-type P2X receptors, and the G protein-coupled metabotropic P2Y receptors. Several subtypes of both P2X and P2Y receptors were found to be expressed by differentiating and mature chondrocytes [20,64]. Upon binding to P2 receptors, ATP may initiate two distinct Ca^{2+} signalling pathways: acting through metabotropic P2Y receptors leads to Ca^{2+} mobilisation from internal stores *via* the PLC–IP_3 pathway, while activation of ligand-gated P2X receptors—that are *bona fide* ion channels—directly enables Ca^{2+} influx. Both pathways lead to the transient elevation of cytosolic Ca^{2+} concentration, eliminated by either active Ca^{2+} pump or exchange functions (sequestration into the ER by SERCA and NCX or into mitochondria by the mitochondrial calcium uniporter or mitochondrial NCX; or extrusion to the extracellular space by the plasma membrane Ca^{2+}-ATPase, PMCA or NCX).

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Second, it should be noted that P2X receptors are non-selective cation channels that mediate Na^+ influx, K^+ efflux and, to a lesser extent, Ca^{2+} influx, leading to cell membrane depolarisation [65]. This then triggers the activation of voltage-operated calcium channels (VOCCs), thus enabling further Ca^{2+} entry across the plasma membrane. VOCCs are also key (albeit not the pivotal) players involved in generation and/or maintenance of repetitive Ca^{2+} transients as their blockade by nifedipine alters the parameters of oscillations, although does not eliminate them. In turn, elevated cytosolic Ca^{2+} may also activate Ca^{2+} activated K^+ channels [66,67], which leads to membrane repolarisation that acts on other ion channels, *e.g.* voltage-dependent potassium channels (K_V). We and others have shown that chondrocytes express mRNA transcripts and proteins of voltage-gated K^+ ion channels [68]; moreover, a switch between $K_V1.1$ and $K_V1.3$ plasma membrane protein expression is required for *in vitro* chondrogenesis [21]. In fact, two cell populations in terms of membrane potential distribution were observed in HDC: in one population, the mean membrane potential was more positive and showed greater fluctuations (-39.0 ± 4.1 mV) while in the other population, cells were much more hyperpolarised with significantly lower cell-to-cell variance (-72.2 ± 1.5 mV) [21]. Periodic alterations in RMP may activate different subsets of VOCCs enabling cyclic Ca^{2+} influx that manifests in periodic Ca^{2+} oscillations. Obviously, other Ca^{2+} channels that enable Ca^{2+} entry across the plasma membrane (*i.e.* NMDA receptors, members of the TRP family, arachidonate-regulated Ca^{2+} channels, *etc.*) may also contribute to maintenance of Ca^{2+} oscillations; however, their detailed analysis is yet to be performed.

The third mechanism may include many signalling pathways (*i.e.* P2Y receptors, *etc.*) that converge on Ca^{2+} release from internal Ca^{2+} stores *via* activation of phospholipase C resulting in the generation of IP_3 which then binds to the IP_3 receptor in the ER membrane and promotes Ca^{2+} release. Store depletion in turn initiates SOCE *via* CRAC channels, which are activated through the binding of the ER Ca^{2+} sensors STIM1 and STIM2 to the CRAC channel protein Orai1 and/or other CRAC channels (for relevant recent reviews, see [27,44]). SOCE seems to be essential for Ca^{2+} oscillations as cells with depleted stores failed to exhibit periodic Ca^{2+} transients. However, Ca^{2+} release alone is insufficient for long-term maintenance of oscillations as transients disappeared when Ca^{2+} entry from the extracellular space was prevented by using Ca^{2+}-free external bath solution. Therefore, SOCE is a key pathway required for sustained Ca^{2+} influx and store replenishment in differentiating chondrocytes.

Amongst other pathways, CaMKII enzyme subtypes, as well as the calcineurin–NFAT signalling axis may be involved in decoding information encoded by high-frequency Ca^{2+} oscillations, thus promoting differentiation of chondroprogenitor mesenchymal cells. Noteworthy that Sox9, the master transcription factor of chondrogenesis and sexual differentiation, can be translocated to the nucleus by binding to calmodulin during sex determination [69], suggesting that this Ca^{2+} dependent pathway may be involved in decoding Ca^{2+}-oscillations in differentiating chondrocytes. However, the precise role of the above mechanisms played in this process is yet to be characterised.

It should be noted that Ca^{2+} entry and Ca^{2+} release are intimately connected and interdependent, and their precise role in generating/maintaining rapid Ca^{2+} oscillations cannot be discussed independently. We therefore hypothesise that the pathways discussed above act in coordination and are all indispensable to normal Ca^{2+} homeostasis of differentiating chondrocytes (although possibly to a variable extent), similarly to what has been described by Kawano et al. in human MSCs [54].

4.6. Concluding remarks

In this study, we provided evidence that rapid transient increases in cytosolic Ca^{2+} concentration are a common feature of cells in chicken primary HDC before and during differentiation (on days 1 and 2). It must be emphasised that these Ca^{2+} oscillations were spontaneous and did not require pharmacological stimulation by agonists, which is a relatively rare phenomenon amongst non-excitable cells where transient increases in cytosolic Ca^{2+} concentration generally occur following exposure to agonists. However, the molecular link between spontaneous high-frequency Ca^{2+} oscillations and regulation of chondrogenesis remains poorly characterised. Nevertheless, Ca^{2+} oscillations were gradually lost as chondroprogenitors became committed chondroblasts and then mature chondrocytes, suggesting they are only characteristic for differentiating cells of HDC. It is generally accepted that Ca^{2+} oscillations are involved in the differentiation of many non-excitable cells, including MSCs and articular chondrocytes. Moreover, the complex interrelation between cyclic changes of ATP and Ca^{2+} levels has also been implicated in these cell types. Although several elements of the pathways involved, including Ca^{2+} sensitive protein kinases and phosphoprotein phosphatases (classic PKC, CaMKII, calcineurin), plasma membrane proteins ($K_V1.1$, $K_V1.3$, VOCC, P2X and P2Y receptors) and transcription factors (CREB, NFAT) have been identified in differentiating chondrocytes by our laboratory and others, the nature of their putative co-operation in generating and maintaining Ca^{2+} oscillations to govern differentiation remains elusive. These findings may prove to be useful for future research aimed at exploiting the regeneration capacity of chondroprogenitor cells found in both healthy [70] and diseased articular cartilage [71]. Extrapolation of our results regarding Ca^{2+} homeostasis of differentiating chondrocytes to cartilage regeneration or bioengineering from human mesenchymal stem cells may also contribute to recent advances in this field.

Conflict of interest statement

All authors disclose that there are neither any financial nor any personal relationships with other people or organisations that could inappropriately influence (bias) their work. There are no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2013.03.003.

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