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SIGNALING AND CELL PHYSIOLOGY

Purinergic signalling is required for calcium oscillations in migratory chondrogenic progenitor cells

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Abstract Osteoarthritis (OA) is the most common form of chronic musculoskeletal disorders. A migratory stem cell population termed chondrogenic progenitor cells (CPC) with in vitro chondrogenic potential was previously isolated from OA cartilage. Since intracellular Ca^{2+} signalling is an important regulator of chondrogenesis, we aimed to provide a detailed understanding of the Ca^{2+} homeostasis of CPCs. In this work, CPCs immortalised by lentiviral administration of the human telomerase reverse transcriptase (hTERT) and grown in monolayer cultures were studied. Expressions of all three IP_3Rs were confirmed, but no RyR subtypes were detected. Ca^{2+} oscillations observed in CPCs were predominantly dependent on Ca^{2+} release and store replenishment via store-operated Ca^{2+} entry; CPCs express both STIM1 and Orai1 proteins. Expressions of adenosine receptor mRNAs were verified, and adenosine elicited Ca^{2+} transients. Various P2 receptor subtypes were identified; $P2Y_1$ can bind ADP; $P2Y_4$ is targeted by UTP; and ATP may evoke Ca^{2+} transients via detected P2X subtypes, as well as $P2Y_1$ and $P2Y_2$. Enzymatic breakdown of extracellular nucleotides by apyrase completely abrogated Ca^{2+} oscillations, suggesting that an autocrine/paracrine purinergic mechanism may drive Ca^{2+} oscillations in these cells. As CPCs possess a broad spectrum of functional molecular elements of Ca^{2+} signalling, Ca^{2+}-dependent regulatory mechanisms can be supposed to influence their differentiation potential.

Keywords SOCE · Calcium oscillation · Osteoarthritis · Mesenchymal stem cell · Chondrogenesis

Abbreviations

2-APB	2-Aminoethoxydiphenyl borate
ARC	Arachidonic acid-regulated Ca^{2+} channel
CPA	Cyclopiazonic acid
CPC	Chondrogenic progenitor cell
CRAC	Calcium release activated Ca^{2+} channel
ESC	Embryonic stem cell
FTHM	Full time at half maximum
HDC	High density cell culture
hTERT	Human telomerase reverse transcriptase
IP_3R	Inositol 1,2,3-trisphosphate receptor
MSC	Mesenchymal stem cell
NCX	Sodium calcium exchanger
OA	Osteoarthritis
PBST	Phosphate-buffered saline with Tween 20
PG	Proteoglycan
PMCA	Plasma membrane Ca^{2+}-ATPase
RyR	Ryanodine receptor
SDS–PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase

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SOCE	Store-operated Ca^{2+} entry
STIM	Stromal interacting molecule

Introduction

The global prevalence of musculoskeletal disorders (comprising more than 200 conditions and syndromes, including various rheumatic, arthritic and joint diseases) is constantly rising owing to unfavourable changes in the population of developed countries (i.e. higher incidence of obesity, increased mean age). Osteoarthritis (OA) is the most common chronic form of conditions affecting articular cartilage. In the initial, largely asymptomatic stages of the disease, the homeostatic balance between cartilage matrix synthesis and degradation is lost [38]. 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 [32]. Indeed, OA-affected cartilage matrix has long been known to undergo profound changes in terms of collagen, glucosaminoglycan and water content [31], which in turn alters the osmolarity of the matrix and its unique ionic milieu, and affects chondrocyte metabolism [30]. While alterations of OA-affected cartilage matrix components and composition have been investigated in detail, the cellular nature of the disease has not been sufficiently characterised.

We have recently documented a distinct cell population with migratory potential in OA-affected articular cartilage [25]. These chondrogenic progenitor cells (CPCs) exhibit certain stem cell surface markers and possess 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 [25].

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. However, knowledge concerning their physiological properties is almost completely lacking. 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 and consequently maintain communication with the extracellular space. Indeed, according to a genome-wide analysis that compared the mRNA transcriptome of chondrocytes isolated from articular cartilage of healthy and OA patients, the expression of several plasma membrane ion channels with a putative role in volume regulation, apoptosis, and proliferation was found to be significantly altered [20].

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. Indeed, NMDA receptors with altered subunit composition and activity were described in OA chondrocytes [44]; furthermore, intracellular calcium oscillations associated with inorganic calcium crystal-induced cartilage matrix degradation were also observed [40]. 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; more specifically, ATP levels can significantly increase following its release by damaged cells [9]. In turn, elevated levels of ATP provoke the activation of purinergic receptors, mainly P2X and P2Y receptors [3]. 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 [52].

In light of these data, we aimed to provide a detailed understanding of the Ca^{2+} homeostasis in undifferentiated, proliferating CPCs immortalised by lentiviral administration of hTERT and grown in monolayer. 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.

Materials and methods

Cell cultures

CPCs were isolated, immortalised and cultured as described earlier [25]. Briefly, adult osteoarthritic cartilage, without signs of rheumatoid involvement, was obtained from the knee joints of patients (ages 65–75 years) suffering from late-stage OA after total knee replacement. Standard explant cultures were performed using 8–15-mm^3 tissue specimens taken from areas adjacent to the main defect. After 10 days, outgrown cells were harvested, and 10^3 cells · cm^{-2} were transferred to a monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA; pH 7.4) with 10 % foetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), supplemented with penicillin/streptomycin (50,000 U/50 mg) and L-glutamine (10 mM), and cultured under standard conditions. Immortalisation of CPCs was carried out by co-transfection of plasmid pLenti6/v5-hTERT with pLP1, pLP2

and pLP/VSVG helper plasmids (ViraPower lentiviral expression system, Invitrogen, Karlsruhe, Germany) into 293FT cells, and the supernatant was collected after 48 h. P1 CPCs were infected with hTERT lentivirus (moi $5 \cdot 10^4$) and selected with 10 mg · mL^{-1} blasticidin for 1 week.

In these experiments, the CPC531 clone cultured in monolayer was used. CPCs were routinely maintained in 75-cm^2 cell culture flasks (Orange Scientifique, Braine-l'Alleud, Belgium) in DMEM containing 1 g · L^{-1} glucose and 10 % FBS until approximately 80 % confluence and were passaged. For calcium measurements, cells were harvested and transferred onto 30-mm round coverslips (Menzel-Gläser, Menzel GmbH, Braunschweig, Germany) placed into 35-mm plastic Petri dishes (Orange Scientifique) at a density of 10^3 cells · cm^{-2}. Cultures were used only until they reached approximately 80 % confluence. For RT-PCR and Western blot analyses, cells were grown in 150-cm^2 tissue culture dishes (Orange Scientifique) until approximately 80 % confluency and were then harvested. The medium was changed on every second day.

To confirm that cells used in this study retained their osteochondroprogenitor nature at the level of their transcriptome, we performed chondrogenic (*Sox9*, *Col2a1*, *Acan*) and osteogenic (*Runx2*, *Col1a1*, *Col10a1*) marker gene expression analysis using RT-PCR (for details, see Table S1 and Fig. S1 given in the Online Resource).

Single cell fluorescent Ca^{2+} measurements

Measurements were performed using the calcium-dependent fluorescent dye Fura-2 as described previously [11]. Briefly, cultures were transferred to 1 mL of fresh DMEM containing 5 μL of Fura-2-acetoxy-methylester (AM; 10 μM; Life Technologies, Carlsbad, CA, USA) and 3 μL of neostigmin (0.3 nM; to inhibit extracellular choline-esterase activity; TEVA, Debrecen, Hungary) 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 (Diaphot; Nikon, Kowasaki, Japan) 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 a 10-Hz acquisition rate using a photomultiplier. Background fluorescence was subtracted online 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 [14]. 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.

Solutions and drugs

Single cell calcium measurements were performed in normal or Ca^{2+}-free Tyrode's solutions (see above). All compounds were purchased from Sigma-Aldrich. The aspecific IP_3R inhibitor 2-aminoethoxydiphenyl borate (2-APB) [2] (75 μM) was diluted in 0 mM Ca^{2+} Tyrode's (stock: 1 M in DMSO); the non-specific TRPC antagonist YM-58483 (a pyrazole derivative; also known as BTP-2) [15] (1 μM) and the aspecific Ca^{2+} influx channel blocker $LaCl_3$ [24] (500 μM) were diluted in normal (1.8 mM Ca^{2+}) Tyrode's solution (stocks: 1 mM and 300 mM in distilled water and DMSO, respectively). The sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA; 10 μM) [27] was diluted in Ca^{2+}-free Tyrode's solution (stock: 10 mM in DMSO). Caffeine (30 mM), which was used in this study to increase the sensitivity of ryanodine receptor (RyR) for calcium, or as an antagonist of P1 adenosine receptors [42], was dissolved in 1.8 mM Ca^{2+} Tyrode's. The general P2X and P2Y purinergic receptor agonist ATP (180 μM) was dissolved in both 0 and 1.8 mM Ca^{2+} Tyrode's; agonists of P2Y purinergic receptors (ADP, UTP and UDP; all 100 μM) were dissolved in 1.8 mM Ca^{2+} Tyrode's. The aspecific P2X and P2Y purinergic receptor inhibitor suramin (10 μM) [43] was dissolved in both 0 and 1.8 mM Ca^{2+} Tyrode's. The P1 receptor agonist adenosine (100 μM) was prepared in 1.8 mM Ca^{2+} Tyrode's. To catalyse the hydrolysis extracellular ATP to AMP, apyrase (5 U · mL^{-1}) diluted in Ca^{2+}-free Tyrode's was applied.

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 (Applied Biosystems, Foster City, CA), and following the addition of 20 % RNase-free chloroform (Sigma-Aldrich) 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 (Promega, Madison, WI, USA) and stored at −70 °C.

The composition of the assay mixture (20 μL) for reverse transcriptase (RT) reactions was as follows: 1,000-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 (High Capacity RT kit; Applied Biosystems, Foster City, CA, USA). cDNA was transcribed at 37 °C for 2 h.

Amplifications of specific cDNA sequences were carried out using specific primer pairs that were designed by Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) based on human nucleotide sequences published in GenBank and purchased from Integrated DNA Technologies, Inc. (IDT; Coralville, IA, USA). The specificity of custom-designed primer pairs was confirmed in silico by using the Primer-BLAST service of NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Nucleotide sequences of forward and reverse primers and reaction conditions are shown in Table S2 in the Online Resource. 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 (Promega) in a programmable thermal cycler (Labnet MultiGene™ 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) 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 (see Table S2 in the Online Resource) 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.

SDS–PAGE and Western blot analysis

Total lysates of CPCs for electrophoresis (SDS–PAGE) were prepared as described previously [11], with minor modifications for subconfluent cell cultures. Briefly, cultures were washed with physiological NaCl solution and were harvested. After centrifugation at 2,000×*g* for 10 min at room temperature, cell pellets were suspended in 100 μL of RIPA homogenisation buffer containing 150 mM NaCl, 1.0 % NP40, 0.5 % sodium deoxycholate, 50 mM Tris, 0.1 % sodium dodecyl sulphate (SDS; pH 8.0), supplemented with protein inhibitors as follows: aprotinin (10 μg · mL^{-1}), 5 mM benzamidine, leupeptin (10 μg · mL^{-1}), trypsin inhibitor (10 μg · mL^{-1}), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 1 mM EGTA, 8 mM Na-fluoride and 1 mM Na-orthovanadate. All components were purchased from Sigma-Aldrich. Samples were stored at −70 °C. Suspensions were sonicated by pulsing burst for three times 30 s by 50 cycles using an ultrasonic homogeniser (Cole-Parmer, Vernon Hills, IL, USA). Samples for SDS–PAGE were prepared by adding 1/5 volume of 5× concentrated Laemmli's electrophoresis sample buffer (310 mM Tris–HCl; pH 6.8, 10 % SDS, 50 % glycerol, 100 mM DTT, 0.01 % bromophenol blue) to cell lysates and heated at 95 °C for 5 min.

Protein 50 μg 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 (Bio-Rad, Hungary). After blocking in 5 % non-fat dry milk in PBS, membranes were incubated with primary antibodies overnight at 4 °C as shown in Table S3 in the Online Resource. After washing for 30 min in PBST, membranes were incubated with the HRP-conjugated secondary antibodies, anti-rabbit or anti-mouse IgG (Bio-Rad) in 1:1,500 dilution. Membranes were developed by enhanced chemiluminescence reaction (Millipore, Billerica, MA, USA) according to the instructions of the manufacturer.

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 $*P<0.05$.

Results

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 1,000-s long intervals. In the majority of cells observed during single cell Ca^{2+} measurements in standard (containing 1.8 mM Ca^{2+}) bath solution (in 20 of 28 cells; 71 %), spontaneous Ca^{2+} transients were observed, with considerable individual variations in frequency and amplitude among cells (Fig. 1a, c, d). 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 2.2 ±0.5 nM · s^{-1} (in each case, $n=20$) (Fig. 1c, d).

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 (e.g. the ER), 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

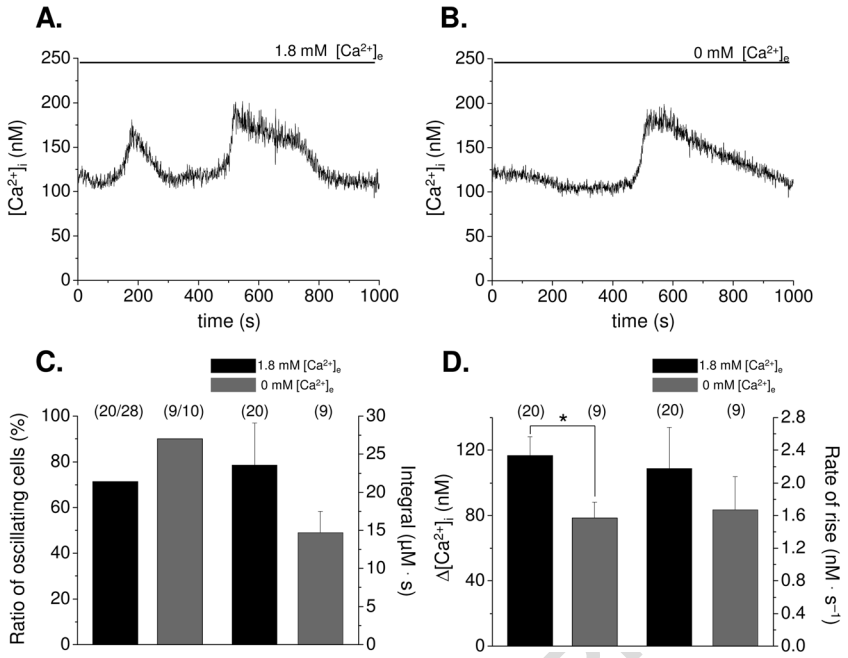

Fig. 1 Spontaneous Ca^{2+} oscillations in CPCs and pooled data demonstrating the different parameters of the phenomenon. **a** Ca^{2+} oscillations detected without agonist stimulation in standard Tyrode's solution containing 1.8 mM Ca^{2+} Tyrode's at room temperature. Representative record of Fura2-AM-loaded cells analysed during 1,000-s long intervals. **b** Ca^{2+} oscillations detected without agonist stimulation in Ca^{2+}-free Tyrode's at room temperature. Representative record of Fura2-AM-loaded cells analysed during 1,000-s long intervals. **c** Ratio of oscillating cells and integral of Ca^{2+} transients. *Numbers* in parentheses above *bars* indicate the number of cells measured. **d** Mean values of the amplitude and the rate of rise of transients (calculated as $d[Ca^{2+}]_i/dt$). *Numbers* in parentheses above *bars* indicate the number of cells measured. *Asterisks* (*) mark significant (*$P<0.05$) differences between cells bathed in standard and Ca^{2+}-free Tyrode's

decreasing amplitudes and frequencies (Fig. 1b, d). 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 released during each Ca^{2+} transient. The rate of rise of transients was 1.7±0.4 nM · s^{-1} (in each case, n=9) (Fig. 1c, d). 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; Fig. 1c, d). 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.

To confirm this claim, further experiments were carried out. First, we investigated to what extent internal stores contributed to the generation of Ca^{2+} spikes. 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 supported 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 cell-permeable 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 implicated 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, nor did it evoke changes in resting Ca^{2+} levels (n=10), suggesting no important regulatory function of RyRs.

Functional characterisation of internal Ca^{2+} stores

Since according to the above results, 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 involved in Ca^{2+} release and re-uptake and found that although all three IP_3R isoforms were observed in CPCs, no specific bands for RyR subtypes could be detected (Table 1). 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

t1.1 **Table 1** mRNA expression of molecules involved in Ca^{2+} homeostasis of CPCs

t1.2	Gene	Band detected at expected amplicon size
t1.3	A. Ca^{2+} release	
Q1 t1.4	*IP_3R Type 1*	+
t1.5	*IP_3R Type 2*	+
t1.6	*IP_3R Type 3*	+
t1.7	RyR 1	–
t1.8	RyR 2	–
t1.9	RyR 3	–
t1.10	B. Store-operated Ca^{2+} entry (SOCE)	
t1.11	*Orai1*	+
t1.12	*Orai2*	+
t1.13	*Orai3*	+
t1.14	*STIM1*	+
t1.15	*STIM2*	+
t1.16	C. Ca^{2+} extrusion	
t1.17	*PMCA1*	+
t1.18	PMCA2	–
t1.19	PMCA3	–
t1.20	*PMCA4*	+
t1.21	*NCX1*	+
t1.22	NCX2	–
t1.23	*NCX3*	+
t1.24	NCX4	–
t1.25	D. Ca^{2+} re-uptake	
t1.26	*SERCA1*	+ (weak)
t1.27	*SERCA2*	+
t1.28	*SERCA3*	+
t1.29	E. P1 purinergic receptors	
t1.30	*A_1*	+
t1.31	*A_{2A}*	+
t1.32	*A_{2B}*	+
t1.33	A_3	–
t1.34	F. P2X ionotropic purinergic receptors	
t1.35	*$P2X_1$*	+
t1.36	$P2X_2$	–
t1.37	$P2X_3$	–
t1.38	*$P2X_4$*	+
t1.39	*$P2X_5$*	+
t1.40	*$P2X_6$*	+
t1.41	*$P2X_7$*	+
t1.42	F. P2Y metabotropic purinergic receptors (G_q-coupled)	
t1.43	*$P2Y_1$*	+
t1.44	*$P2Y_2$*	+
t1.45	*$P2Y_4$*	+
t1.46	*$P2Y_6$*	+
t1.47	*$P2Y_{11}$*	+

Orai (Orai1, Orai2 and Orai3) mRNAs were expressed by CPCs (Table 1).

Having confirmed the presence of mRNA transcripts of these molecules, the next step was to identify their protein level expression. By applying isotype-specific IP_3R anti-sera, all IP_3R subtypes (IP_3R1, IP_3R2 and IP_3R3) were found to be expressed by CPCs as revealed by Western blot analyses (Fig. 2a). Of the molecules that orchestrate SOCE, only STIM1 and Orai1 were detectable at the protein level (Fig. 2a).

The functionality of these proteins was investigated during single cell fluorescent Ca^{2+} recordings (Fig. 2b, d). When the SERCA blocker CPA (10 μM) was administered to cells in Ca^{2+}-free Tyrode's, an approximately 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 (i.e. plasma membrane Ca^{2+} ATPase, PMCA or Na^+–Ca^{2+} exchanger, NCX; mRNA expressions are shown in Table 1). 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). This data is in a good correlation with what has been described in various other cell types [4]. 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 in response to depleted internal stores, demonstrating store-operated Ca^{2+} entry (SOCE; Fig. 2b, c). 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 ($\Delta[Ca^{2+}]_i$: 131.0±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 (2.0±0.5 nM · s^{-1} vs. 0.9±0.2 nM · s^{-1}; n=13 and 8; P=0.16) were not statistically significant (Fig. 2b, d). This is probably a consequence of large individual variations among cells.

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

Given that our results implicate the dependence of spontaneous fluctuations in cytosolic Ca^{2+} on the activation of internal Ca^{2+} stores via IP_3R, we aimed to identify signalling pathways that couple extracellular signals to the IP_3R. Since periodic Ca^{2+} transients in human mesenchymal stem cells (MSCs) could be attributed to an autocrine/paracrine purinergic signalling loop [22], and as the ionotropic purinoreceptor $P2X_4$ was proved to play a role in differentiation of chicken chondroprogenitor cells [10], we hypothesised the presence of a similar purinergic signal transduction mechanism also in migratory chondroprogenitor cells. To investigate the possibility of such regulatory systems, we first carried out a comprehensive mRNA transcript screening for all purinergic

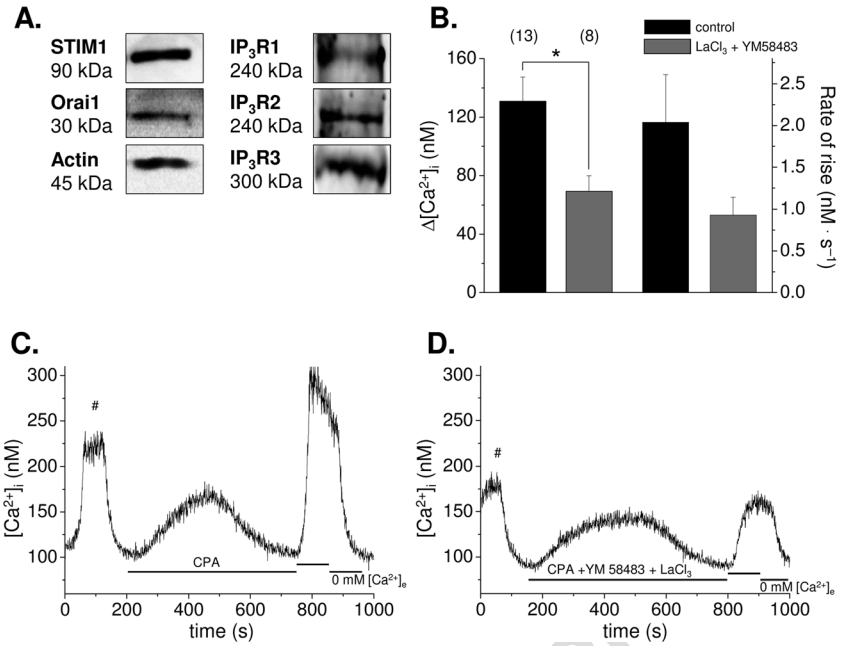

Fig. 2 Functional investigation of internal Ca^{2+} stores in CPCs. **a** Protein expression of molecules (STIM1 and Orai1) that orchestrate the SOCE mechanism and isotypes of the Ca^{2+} release channel of internal stores (IP_3R1, IP_3R2 and IP_3R3). Actin was used as a control. Representative data of three independent experiments. **b** Pooled data of amplitudes and rate of rise of transients evoked by Ca^{2+} entry after the re-administration of external calcium. *Numbers* in parentheses indicate the number of cells measured. *Asterisks* (*) mark significant (*$P<0.05$) differences between the control and cells pre-treated with $LaCl_3$ and YM-58483. **c** Store-operated Ca^{2+} entry (SOCE) in Fura-2-loaded CPCs 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 500–600 s, which caused a prominent increase in $[Ca^{2+}]_i$. When $[Ca^{2+}]_e$ was changed back to normal (1.8 mM), a large transient increase in $[Ca^{2+}]_i$ was observed, demonstrating SOCE. Representative record. *Hashmark* (#) indicates spontaneous Ca^{2+} transients at the beginning of measurements. **d** Store-operated Ca^{2+} entry (SOCE) in Fura-2-loaded CPCs during single cell fluorescent Ca^{2+} imaging in the presence of SOCE inhibitors 500 μM $LaCl_3$ and 1 μM YM-58483. *Hashmark* (#) indicates spontaneous Ca^{2+} transients at the beginning of measurements. Representative record

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-coupled P2Y metabotropic purinergic receptor subtypes were detectable using RT-PCR (see Table 1; mRNA expression of other P2Y receptor subtypes are shown in the Online Resource in Table S4 and Fig. S2).

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 (Fig. 3a; Table 2). The average duration of transients expressed as full time at half maximum (FTHM) values was 16.1±2.6 s. When ATP was administered in 0 mM external Ca^{2+}, repetitive transients could still be evoked ($n=35$ of 36 cells; 97 %; Fig. 3b), but with significantly lower amplitudes compared to the control (204.6±25.0 nM; $n=35$; $P=0.0009$; Fig. 3d). Similarly, there was a 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}; $P=0.02$; Fig. 3e). However, no change was observed in the duration of transients (20.6±3.1 s; $P=0.29$).

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; 10 %; Fig. 3c), raising the possibility that mainly metabotropic P2Y receptor activity could be accounted for the observed Ca^{2+} transients or that 2-APB could aspecifically block Ca^{2+} influx. To confirm these hypotheses, further experiments were conducted.

Having suggested the key role of P2Y receptors in the Ca^{2+} homeostasis of CPCs, we undertook to further characterise their precise involvement in generating Ca^{2+} transients upon agonist stimulation. To this end, the effects of various P2Y receptor agonists were tested during single cell Ca^{2+} measurements (see Table 2). While UTP (100 μM) and ADP (100 μM) evoked prominent Ca^{2+} transients (324.5±20.5 and 284.0±25.0 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

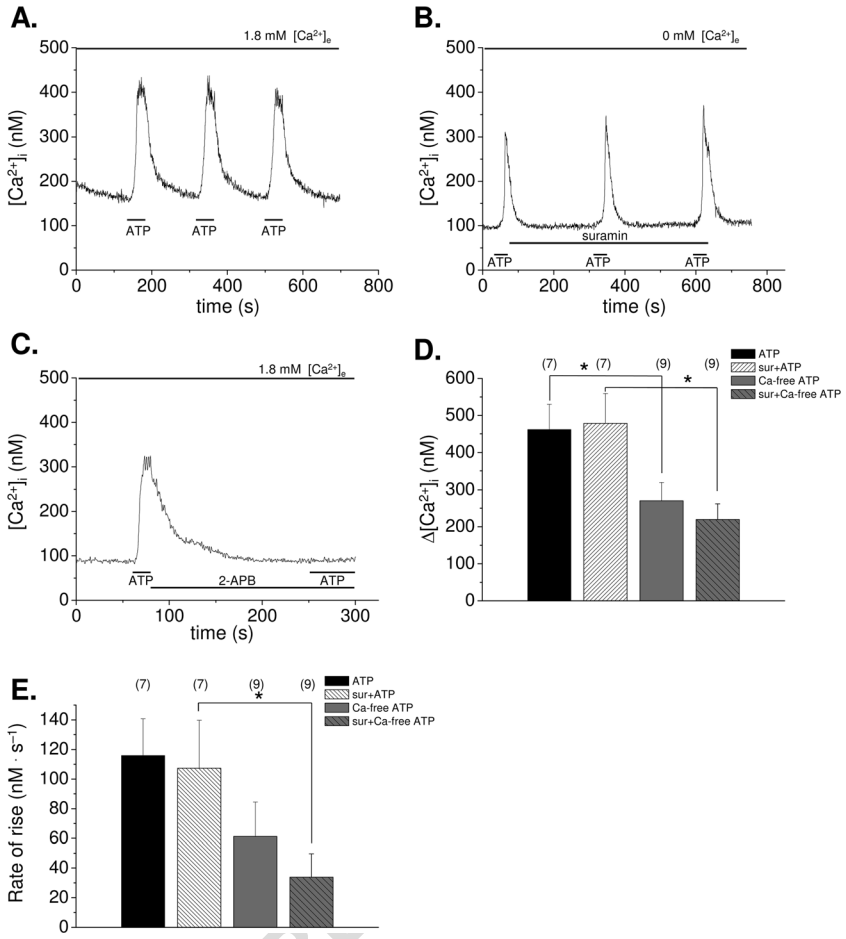

Fig. 3 Functional characterisation of P2 purinergic receptors in CPCs. **a** Calcium transients elicited by repeated administration of ATP in the presence of external calcium. Representative record. **b** Effect of the non-specific P2 purinergic antagonist suramin (10 μM) on ATP-evoked calcium transients in the absence of external calcium. Representative trace. **c** Representative record showing the lack of ATP-evoked Ca^{2+} transients in the presence of the aspecific IP_3R inhibitor 2-APB in 1.8 mM Ca^{2+}-containing Tyrode's. **d** Changes in the amplitude of ATP-evoked calcium transients detected in 1.8 mM Ca^{2+}-containing and Ca^{2+}-free Tyrode's. Ca^{2+} transients recorded before suramin application were considered as controls. *Numbers* in parentheses show the number of cells measured. *Asterisk* (*) marks a significant (*$P<0.05$) difference between cells bathed in standard and Ca^{2+}-free solutions. **e** Changes in the rate of rise of ATP-evoked calcium transients detected in 1.8 mM Ca^{2+}-containing and Ca^{2+}-free Tyrode's. Ca^{2+} transients recorded before suramin application were considered as controls. *Numbers* in parentheses show the number of cells measured. *Asterisk* (*) marks a significant (*$P<0.05$) difference between cells bathed in standard and Ca^{2+}-free solutions

(n=0 of 11 cells; Fig. 4a, c, e). Furthermore, the functionality of P1 adenosine receptors was also confirmed as local application of adenosine (100 μM) evoked Ca^{2+} transients (average amplitude 258.5±26.7 nM) in most of the cells

t2.1 **Table 2** Minimum (resting) and maximum Ca^{2+} concentration values, transient amplitudes and transient durations expressed as full time at half maximum (FTHM) values during agonist stimulation (ATP, 180 μM; ADP, 100 μM; UTP, 100 μM; Ade, 100 μM) of CPCs

t2.2		$[Ca^{2+}]_{i,\ min}$ (nM)[a]	$[Ca^{2+}]_{i,\ max}$ (nM)[a]	Amplitude (nM)[a]	FTHM (s)[a]
t2.3	ATP, 180 μM ($[Ca^{2+}]_i$=1.8 mM), n=44	121.2±3.5	452.8±28.9	338.2±28.6	16.1±2.6
t2.4	ATP, 180 μM ($[Ca^{2+}]_i$=0 mM), n=35	97.0±4.2	301.7±27.0	204.6±25.0	20.6±3.1
t2.5		$P=2.8 \cdot 10^{-7}$*	P=0.0002*	P=0.0009*	P=0.29*
t2.6	ADP, 100 μM ($[Ca^{2+}]_i$=1.8 mM), n=18	112.0±3.1	396.1±24.9	284.1±25.0	24.1±2.5
t2.7	UTP, 100 μM ($[Ca^{2+}]_i$=1.8 mM), n=13	98.2±3.9	422.8±23.2	324.6±20.50	23.0±2.6
t2.8	Ade, 100 μM ($[Ca^{2+}]_i$=1.8 mM), n=16	119.6±11.5	378.1±34.6	258.5±26.7	23.0±3.2

*P values refer to differences between ATP transients elicited in Tyrode's containing 0 mM vs. 1.8 mM Ca^{2+} (Student's t test)

[a] Data are expressed as mean ± standard error of the mean (SEM)

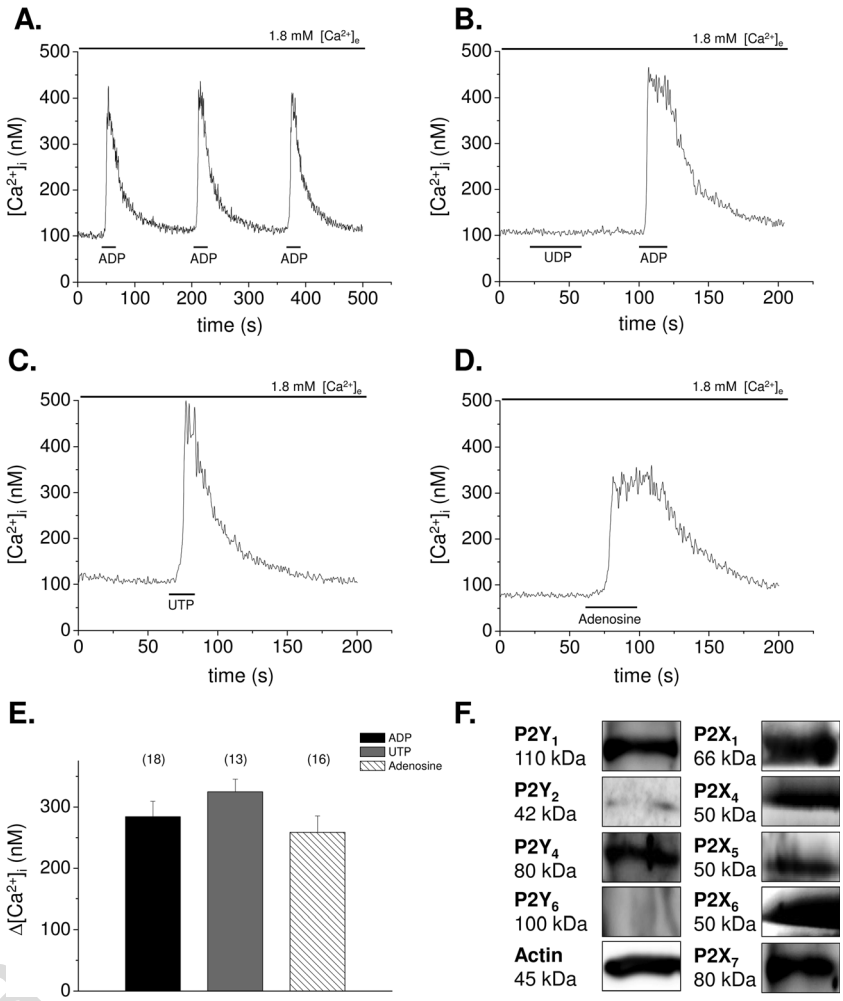

Fig. 4 Functional characterisation of P1 and P2Y receptors. **a** Effect of repetitive application of 100 μM ADP on Ca^{2+} transients measured in the presence of external calcium. Horizontal lines indicate the application of ADP. Representative trace. **b** Representative record showing that 100 μM UDP was ineffective to evoke Ca^{2+} transients in the presence of external calcium. **c** Calcium transient elicited by administration of UTP in the presence of external calcium. Representative trace. **d** 100 μM adenosine-evoked Ca^{2+} transient measured in the presence of external calcium proving the functionality of A1 receptors in CPCs. Representative record. **e** Average values of amplitudes for each nucleotide-evoked Ca^{2+} transient. Data represent mean ± standard error of the mean (SEM). *Numbers* in parentheses above *bars* indicate the number of cells measured. **f** Western blot analysis of purinergic receptor subtypes. Total protein samples were used (30 μg in each lane) to examine the protein expression level. Actin was used as a control. Representative data of three independent experiments

investigated (n=16 of 18 cells; Fig. 4d), while co-application of adenosine with the P1 receptor blocker caffeine (30 mM) eliminated this response (n=0 of 10 cells). The average duration of transients elicited by these agonists was approximately the same. Table 2 summarises the main parameters of agonist-induced transients (i.e. minimum and maximum cytosolic Ca^{2+} concentrations, amplitudes and durations expressed as FTHM values).

Functional expression of P2 purinergic receptors

As mentioned earlier, mRNAs of various P2 purinergic receptor subunits were detected in the transcriptome of CPCs (see Table 1). 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 (Fig. 4f). Uncropped Western blot images are shown in the Online Resource (Figs. S3, S4 and S5).

To rule out the function of certain P2X and P2Y receptors, the non-specific P2 receptor antagonist suramin (10 μM) was administered prior to and during local application of P2 receptor agonists. As seen in Fig. 3b, d, e, none of the parameters of Ca^{2+} transients evoked by ATP either in 1.8 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.

Since Ca^{2+} oscillations in hMSCs are known to be mediated by an autocrine/paracrine purinergic signalling loop, we also wanted to confirm whether repetitive Ca^{2+} transients in CPCs were linked to the function of purinergic receptors. 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 · 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.

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Discussion

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

Repetitive increases of cytosolic $[Ca^{2+}]$, also referred to as Ca^{2+}, oscillations represent a nearly universal signalling mechanism in non-excitable cells by reducing the threshold for the activation of calcium-dependent transcription factors such as NFAT, NF-κB or CREB [8] and by modulating protein kinase activity [26, 34]. Ca^{2+} oscillations have been reported in a great variety of non-excitable cells including pancreatic β-cells [49], embryonic stem cells (ESCs) [47], chondroblasts [11, 35], chondrocytes [40] and human MSCs [22, 24]. This is the first study to report that human chondroprogenitor cells derived from regions with minor lesions of osteoarthritic articular cartilage (CPCs) are also characterised by a similarly dynamic Ca^{2+} homeostasis. It is of note that these cells were immortalised by viral transfection of hTERT and are characterised by continuous proliferation under monolayer culturing conditions applied in these experiments. Nevertheless, the chondrogenic potential of these cells has been demonstrated [25]; and here, we present data that the mRNA expression of various osteochondrogenic lineage markers was detectable in CPCs. In contrast to embryonic chicken limb bud-derived chondrogenic cells where high-frequency (8×10^{-2} Hz) Ca^{2+} oscillations could be detected during fluorescent single cell Ca^{2+} measurements [11, 53], CPCs exhibited less frequent (1.3×10^{-3} Hz) periodic increases in $[Ca^{2+}]_i$, similar to what has been described in MSCs [24, 50]. Likewise, Ca^{2+} oscillations in CPCs proved to be driven by internal Ca^{2+} stores as they could still be detected in Ca^{2+}-free medium, with decreasing amplitudes. Noteworthy that rapid Ca^{2+} oscillations in embryonic chicken limb bud-derived chondroprogenitor cells were primarily mediated by extracellular Ca^{2+}, probably due to their much smaller internal Ca^{2+} stores [11, 53].

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. In terms of the two major Ca^{2+} release channels of the ER, CPCs share homology with MSCs wherein all IP_3R subtypes but no RyR isoforms are expressed [24, 50], as opposed to chicken limb bud-derived chondroprogenitor cells wherein only IP_3R1 could be detected [33]. ESCs, on the other hand, are known to express all three IP_3R subtypes [19], as well as RyRs [47]. The fact that CPCs express all three IP_3R subtypes is not surprising as IP_3Rs are known to be key components of Ca^{2+} oscillations and most cells and tissues examined possess more than one subtype. In fact, the properties of Ca^{2+} oscillation patterns depend on the relative expression levels of IP_3R subtypes, probably because of their specific response to endogenous modulators, such as IP_3, Ca^{2+} and ATP. Pertaining to Ca^{2+} oscillations, IP_3R2 is required for robust, long-lasting and regular Ca^{2+} oscillations; IP_3R1 mediates less regular Ca^{2+} oscillations; and given that IP_3R3 is the least sensitive to IP_3 as well as to Ca^{2+}, it generates only monophasic Ca^{2+} transients [37, 54]. Contribution from all IP_3R subtypes is likely necessary for the characteristics (frequency, amplitude, amount of liberated Ca^{2+}, etc.) of Ca^{2+} oscillations in CPCs.

The indispensable role of IP_3R-mediated Ca^{2+} release from internal Ca^{2+} stores in the generation of cytosolic Ca^{2+} oscillations was further supported by the fact that application of the aspecific IP_3R blocker 2-APB during single cell Ca^{2+} measurements completely abolished spontaneous Ca^{2+} events. These results are in perfect agreement with what has been observed in MSCs [19, 23, 24] and also in ESCs [19], suggesting that this might be a universal mechanism in the generation of Ca^{2+} oscillations in stem cells. Moreover, we provided evidence that SOCE was also essential for Ca^{2+} oscillations in CPCs.

Our hypothesis that Ca^{2+} oscillations in CPCs were predominantly dependent on Ca^{2+} release through IP_3R followed by store replenishment via SOCE was supported by key data showing that the amount of Ca^{2+} liberated during spontaneous Ca^{2+} oscillations was almost identical to Ca^{2+} released during CPA-induced store depletion. To our best knowledge, such an intimate interrelation between Ca^{2+} oscillations and the Ca^{2+} content of internal Ca^{2+} stores has not been documented in stem cells.

The important role of SOCE in the Ca^{2+} homeostasis as well as the presence of STIM1 and Orai1 in MSCs have been documented [17, 24]; furthermore, the requirement of SOCE to rapid Ca^{2+} oscillations along with STIM1, STIM2 and Orai1 expressions have also been reported in chicken chondroprogenitor cells [11]. The fact that 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 (EC_{50}=406 mM vs. 210 mM for STIM1), and therefore, it is the primary isoform that gets activated at the initial steps of store depletion, while STIM1 only becomes active once the stores are almost fully emptied [16]. Given that a considerable amount of Ca^{2+} is released during each spontaneous transient from the internal stores, it is primarily STIM1 that may play a determining role in triggering Orai isoforms [1].

As far as Orai isoforms are concerned, we confirmed that CPCs express Orai1 proteins only. The fact that Orai1 and Orai2 share many similarities may explain why CPCs are devoid of that isoform [16]. Lack of Orai3, an essential component of the store-independent, arachidonic acid-regulated Ca^{2+} (ARC) channels, raises the possibility that ARC channels are also absent from these cells [36]. The distinct properties of Orai isoforms have recently been

reviewed by Hoth and Niemeyer [16]. However, a detailed characterisation of the precise role of STIM1 and Orai1 and their function in generating Ca^{2+} oscillations and their potential consequences of lineage-specific differentiation of CPCs require further analyses.

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

Ca^{2+} oscillations in human MSCs have been reported to be driven by an autocrine/paracrine purinergic loop, whereby ATP released through hemi-gap junctions triggered $P2Y_1$ metabotropic purinergic receptors and associated PLC-β and IP_3R signalling [22]. More recently, we have implicated a similar autoregulatory pathway in differentiating cells of chicken chondrogenic cell cultures, with $P2X_4$ ionotropic purinergic receptor being the most likely candidate [10, 11]. Given that CPCs were originally derived from OA-affected articular cartilage wherein extracellular nucleotides released along with inflammatory mediators may be considered as important ligands of purinergic receptors [41], we aimed to study whether CPCs were able to respond to nucleotide stimulation by P1 and/or P2 receptor activation.

First, we established the P1 purinergic receptor profile of CPCs, which correlated well with that of MSCs as they are reported to express A_1 [7], A_{2A} [21] and A_{2B} [46], but no A_3 receptors. In particular, A_1 and A_{2B} receptor-mediated signalling is known to be required for osteogenic differentiation of MSCs [7, 48]. In a different study, Gharibi and colleagues demonstrated that the activation of A_{2B} receptors was required for the osteogenic gene induction of MSCs, whereas adipogenic differentiation was accompanied by significant increases in A_1 and A_{2A} receptor expression [12]. The same P1 receptor subtypes were found to be expressed in articular chondrocytes [45]. The ability of A1 purinoreceptors to induce differentiation towards various lineages may reflect on specific intracellular downstream mechanism linked to distinct P1 receptor subtypes. For further details, the authors refer the reader to a recently published review article in this field [5].

Next, we analysed the P2 purinergic receptor expression in CPCs. Ca^{2+} transients elicited by extracellular ATP administration during fluorescent single cell Ca^{2+} measurements were significantly smaller in Ca^{2+}-free medium compared to normal Ca^{2+}-containing solution as shown in Table 2, suggesting the involvement of both P2X and P2Y receptor isotypes. By

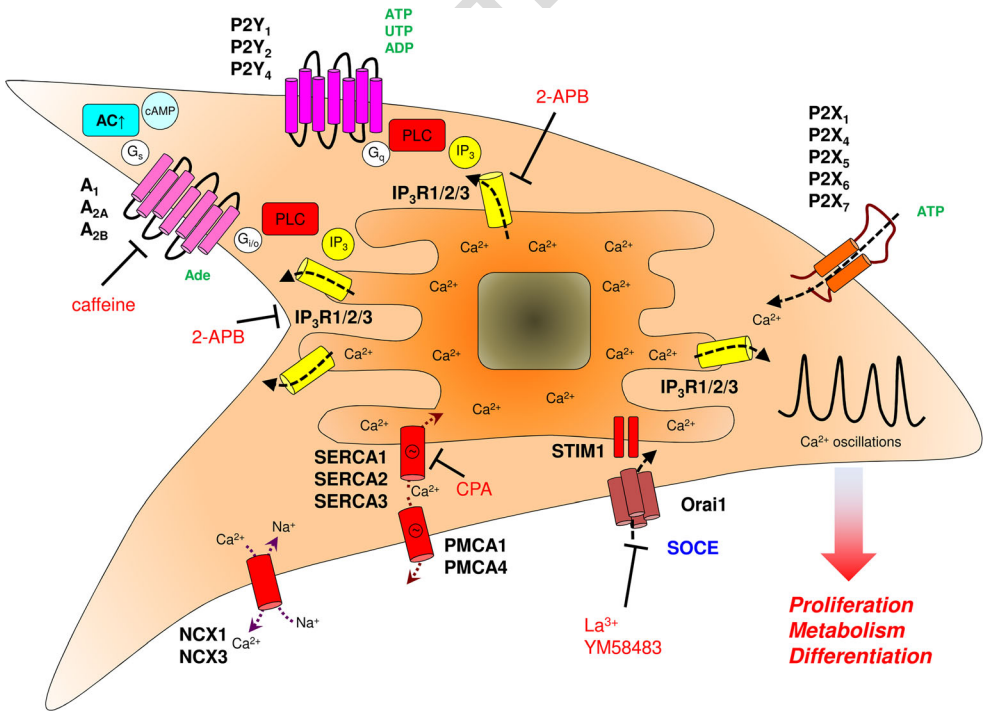

Fig. 5 Diagrammatical representation of molecular components of the Ca^{2+} toolkit verified in immortalised CPCs. Two main types of Ca^{2+} channels were identified; P2X ionotropic purinergic receptors and Orai1 CRAC channels. Of the two types of Ca^{2+} release channels, only IP_3R1, 2 and 3 were verified, but no RyR expression and function was detected. Metabotropic P1 and P2Y receptor subtypes coupled to Ca^{2+} release were also identified. All three major Ca^{2+} elimination pathways (SERCA, PMCA, NCX) were detected at least at the mRNA level. STIM1 ER Ca^{2+} sensor protein was verified, and the functionality of SOCE was also recorded. Ca^{2+} oscillations are driven by external nucleotide (ATP) stimulation and maintained by Ca^{2+} release through IP_3R subtypes and subsequent Ca^{2+} influx (SOCE). Extracellular nucleotides may be released by CPCs in vitro and/or along with inflammatory mediators in osteoarthritic articular cartilage in vivo where other cell types can also be the source of their release (e.g. synovial cells; see [29]). Ca^{2+} oscillations may regulate proliferation, metabolic activity and differentiation of CPCs. Receptor agonists are shown in *green*, and antagonists are in *red* colour

contrast, when ATP was co-applied with the IP_3R inhibitor 2-APB, the majority of cells was unresponsive, implying that the primary mediators of extracellular nucleotides were metabotropic P2Y receptors. However, due to the apparent aspecificity of this inhibitor, the contribution of P2X receptors cannot be fully excluded [2]. By using various P2 receptor agonists (ATP, ADP, UTP and UDP; see Table 2 and [18]), as well as the non-specific receptor antagonist suramin, our results suggest the function of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_4$ and $P2X_6$ receptor subtypes in CPCs. These results are in a very good correlation with the findings of Zippel and colleagues who implicated the P2 receptor subtypes $P2X_6$, $P2Y_4$ and $P2Y_{14}$ to be key regulators in MSC commitment [55]; most of these receptors were active also in CPCs. Furthermore, these P2 receptor subtypes were found to be regulated during both adipogenic and osteogenic differentiation [55], implying a similar role of purinergic receptors in the differentiation of CPCs. The purinergic concept of MSC differentiation was further supported by the recently published results of Ciciarello and colleagues, who demonstrated that adipogenic differentiation of MSCs was mainly mediated by $P2Y_1$ and $P2Y_4$ receptors, while stimulation of the A_{2B} adenosine receptor subtype was primarily involved in osteogenic differentiation [6]. Given that CPCs can differentiate to osteogenic, adipogenic and chondrogenic lineages [25], their P1 and P2 receptor profile is in good correlation with the above data.

Purinergic receptors are known to mediate Ca^{2+} oscillations in various cell types [10, 13, 22, 28]. In this work, we propose that purinergic signalling may be the main drive Ca^{2+} of oscillations in CPCs as (1) breakdown of extracellular ATP and ADP by apyrase was able to completely abrogate Ca^{2+} oscillations and (2) 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. Furthermore, the requirement of Ca^{2+} oscillations for the differentiation of CPCs is likely as the differentiation of MSCs has been reported to be directly influenced by both the application of P2 receptor agonists/antagonists and apyrase-induced nucleotide cleavage [55]. This, however, was beyond the scope of the current work and is yet to be determined.

Conclusions and perspectives

This is the first study to provide a functional characterisation of the Ca^{2+} toolkit in migratory chondroprogenitor cells derived from osteoarthritic cartilage (Fig. 5). It must be noted that the chondrogenic progenitor cell population (CPCs), which served as the cellular source of investigations described in this study, have been artificially immortalised by lentiviral introduction of the hTERT gene. Still, this cell population has been shown to undergo chondrogenic differentiation under appropriate culturing conditions [25]. In this paper, we provided evidence that CPCs are characterised by a dynamic Ca^{2+} homeostasis; we concluded that an extensive purinergic receptor array may drive Ca^{2+} oscillations that also intimately rely on Ca^{2+} release from internal Ca^{2+} stores through IP_3R subtypes and store refilling via SOCE. In many ways, the Ca^{2+} homeostasis of CPCs exhibit an intermediate stage between MSCs and chondroblasts/osteoblasts, which sets them apart from these cell populations. The P1 and P2 purinoreceptor profiling of CPCs revealed that they functionally express many receptor subtypes that are known to be involved in regulating Ca^{2+} oscillations and subsequent activation of transcription factors (e.g. NFAT, CREB), as well as commitment towards various lineages, metabolic activity and proliferation. Thus, the molecular components of the Ca^{2+} signalling toolkit identified in this work (Fig. 5) may provide CPCs with a framework that controls their commitment and subsequent differentiation. It is plausible to assume that through the activation of Ca^{2+} and other ion channels, Ca^{2+} signalling eventually converges on transcription factors such as Sox9 and/or Runx2, as well as proliferation. Such a connection has been verified during chondrogenesis [10, 11, 39, 51].

Given that OA is a currently incurable disease, any attempt to hinder or reverse disease progression and thus augment symptoms of millions of patients suffering from this condition are of utmost importance. To develop novel, targeted therapeutic approaches, a better understanding of the cellular background of the disease is inevitable. Notwithstanding their chondrogenic potential, CPCs fail to differentiate into chondroblasts in situ to initiate repair, probably owing to the non-permissive milieu of inflammatory cartilage. Having provided a detailed description on important aspects of Ca^{2+} signalling in CPCs, targeted alterations of their signal transduction pathways may enable us to exploit their chondrogenic differentiation potentials.

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Conflict of interest 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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Q1. Please provide significance for the items in italic (originally bold). Otherwise, please remove the emphasis.
Q2. Please check if the edit to the sentence starting with "Repetitive increases of cytosolic [Ca2+], also referred to as Ca2+, oscillations…" is correct or if the intended meaning of the text is retained.