SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Comparative analysis of chondrogenic models and investigation of calcium homeostasis in differentiating chondrocytes

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The Examination takes place at the Department of Medical Chemistry, Faculty of Medicine, University of Debrecen at 11 a.m., 31st of July, 2020.

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The PhD Defense takes place at the Lecture Hall of In Vitro Diagnostic Centre, Faculty of Medicine, University of Debrecen at 1 p.m., 31st of July, 2020.

INTRODUCTION

Musculoskeletal disorders comprise a set of diseases that affect a major portion of the population at various stages throughout their life. Typically, these conditions include alterations that cause a significant deterioration in one's quality of living, particularly in the case of osteoarthritis or discopathy; with the latter one also attributed as a nervous system disease. Severe forms of the above musculoskeletal diseases are strongly age- and weight-related, which – in the light of current tendencies in the relevant composition of modern Western societies – is something that makes them an even more critical issue for the future. The World Health Organization of the United Nations recognized the stake of this matter and launched an independent global non-profit organization, the Bone and Joint Decade for the period between 2000 and 2010 to reduce the burden and cost of musculoskeletal conditions to individuals, careers and society worldwide. The organization has been remandated in 2010 as the Global Alliance for Musculoskeletal Health owing to the importance of their job and the long-continued nature of the crisis. A vital pillar in reaching these dignified goals is carrying out cutting-edge research aimed at understanding basic biological processes of the involved tissues, such as pathways and genetic factors that govern various functions of developing and mature articular cartilage.

Our research group at the Department of Anatomy, Histology and Embryology has several decades of experience in studying *in vitro* chondrogenesis. As it is reflected in the following work, we are putting effort into expanding the range of our experiments to multiple chondrogenic models. Moreover, we are also working on gathering data on the role of certain genes in cartilage development from *in vivo* experiments with conditional knockout (CKO) mice. Although there are examples where chondrogenic differentiation of the C3H10T1/2 cell line and primary limb bud-derived micromass cultures has been analyzed, we concluded that an exhaustive comparison under well-standardized conditions, which takes into account mRNA expression profiles of numerous genes that are characteristic for differentiation in the trilineage mesenchymal (i.e., chondro-, osteo- and adipogenic) direction in an array of

models would fill an important gap in our knowledge; enabling simultaneous comparison of mouse and chicken, plus primary and cell line-based chondrogenic models.

To this day, high density cultures (HDC) established from chondroprogenitor mesenchymal cells gained from limb buds of chicken embryos in Hamburger-Hamilton developmental stages 22-24 (4.5-day-old) are among the most universal representatives of chondrogenic models. The most prominent advantage of this model is that chondrogenic differentiation takes place spontaneously under proper experimental conditions and high cellular density; a significant amount of hyaline cartilage is produced and organized into nodules by day 6 of culturing. The ideal nature of this model and the rationale behind its use are further supported by comparative results presented below, hence, experiments presented in the other part of this work are based on investigations using these cultures.

Calcium ions are key second messengers in numerous physiological processes of cellular organisms. Electromechanical coupling in skeletal muscle is one of the earliest-described roles of Ca^{2+}. Since then, numerous other functions have been uncovered, a non-exhaustive list of these includes regulation of the cell cycle, differentiation, gene expression, apoptosis, enzymes, exocytosis/neurotransmitter release and cellular response to hormones. Temporal and spatial features of the Ca^{2+} signal are precisely regulated, since the duration and subcellular localization of these cellular events display incredible variability. It has been proven that Ca^{2+} also plays a pivotal role in chondrogenesis as a second messenger, however, there are numerous molecules and processes linked to the Ca^{2+} homeostasis of differentiating chondrocytes that are not known exactly.

Therefore, the other part of the work was to further explore Ca^{2+} signaling during cartilage development using the chicken primary HDC model. In the recent years, our laboratory has successfully enriched the available literature on chondrogenesis, with special regards to intracellular Ca^{2+} events, related signaling molecules and pathways. Our findings that verify the presence of voltage-dependent Ca^{2+} channels (VDCC) and molecular components that contribute to store-operated Ca^{2+} entry (SOCE), their role in

generating and maintaining high-frequency repetitive Ca^{2+} transients and thus chondrogenic differentiation, are presented below. In our opinion, this is highly important, as future cartilage-healing approaches will surely need to be based on a detailed knowledge of signaling events of how a mesenchymal cell differentiates into a mature chondrocyte, in which – with contribution of works from our laboratory – Ca^{2+} is already known as a prominent secondary messenger of chondrogenic intracellular events.

Therefore this work is an attempt to synthesize our results gathered in a hard-to-define field that simultaneously draws merit from embryology, biochemistry and physiology into a work that will hopefully contribute to the advancement towards the Holy Grail of cartilage research, an ideal cure for chondropathies. Included in this work is a comparative analysis of notable chondrogenic models, as well as novel developments and factors in the relationship of intracellular Ca^{2+} concentration changes and chondrogenesis.

Aims and Objectives

Accordingly, our aims and objectives were to find answers for the following questions:

1. How will mRNA-levels change throughout an extended culturing period during an expression analysis of a well-selected pool of marker genes in control and BMP-2 overexpressing C3H10T1/2 (marked as c- and b-C3H10T1/2, respectably, in the rest of this work) micromass cultures, as well as mouse and chick embryonic limb bud-derived micromass cultures?
2. What will differentiation potentials in osteo- and chondrogenic directions reflect in the above models based on marker gene mRNA levels? Are there cells undergoing adipogenic differentiation in the colonies?
3. Will morphological observations following histological staining procedures that are suitable for determining cellular morphology, chondrogenic differentiation, matrix mineralization and lipid accumulation in high density cultures (HDC) determine any difference in the examined models? If yes, what are these differences? Are these results coherent with those obtained from investigating marker gene expression levels?
4. Are there cells that maintain a pluripotent state in HDC? If so, is there a difference in the expression of pluripotency markers among the models?
5. Which model(s) are the most suitable for conducting research on chondrogenesis (if there is an unequivocally superior one at all)? What are some of the main practical considerations?
6. Do the differentiating chondrocytes of our chicken model possess VDCCs and the molecules that are required for SOCE?
7. What is the input of internal Ca^{2+} stores and the involvement of Ca^{2+} release-activated Ca^{2+} channels (CRACs) and VDCCs in generating and maintaining high-frequency repetitive Ca^{2+} transients to

modulate cellular functions such as differentiation, metabolic activity and proliferation?

8. What kind of dynamics can be characterized at high spatial and temporal resolution by analyzing intracellular Ca^{2+} transients of individual chondrifying cells using LIVE confocal Ca^{2+} imaging microscopy? Can the observed dynamics be modified by adjusting the extracellular ionic milieu or by the application of SOCE and/or VDCC blockers?
9. Will we be able to observe altered parameters in store depletion-induced SOCE following application of SOCE blockers in single cell Ca^{2+} measurements?
10. What kinds of alterations, if any, can we notice on such cellular parameters as differentiation, metabolic activity and proliferation of chondrogenic chick HDC following the application of an inhibitor of septin rearrangement, which – according to recent research – is of key importance in the activation of CRACs?
11. In conclusion, the final aim of this work is to provide a refined model of Ca^{2+} signaling events including Ca^{2+} influx and release functions with the inclusion of a recently uncovered regulator of SOCE in the well-established chicken chondrifying HDC during *in vitro* chondrogenesis.

Materials and Methods

Cell Culturing

C3H10T1/2 is an embryonic murine mesenchymal stem cell line that was purchased from the American Type Culture Collection. We have also obtained a modified version of this cell line that has been permanently transfected with the eukaryotic expression vector pMBC-2T-fl containing the cDNA encoding the human bone morphogenic protein BMP-2 as a kind gift from G. Gross. Culturing of cells was performed in high glucose Dulbecco's modified Eagle's medium (DMEM). The culture medium of the b-C3H10T1/2 cells contained 5 $\mu g \cdot mL^{-1}$ puromycin to provide selective pressure to maintain consistent expression. Cultures were incubated in a humidified CO_2 incubator at 37 °C. Cells were passaged at 80% confluency. To establish C3H10T1/2 micromass cultures, cellular density was counted and set at 1.5×10^7 $cells \cdot mL^{-1}$. In all cases, the day of plating was considered day 0 of culturing. To establish chicken primary micromass cell cultures, Ross hybrid chicken embryos of Hamburger-Hamilton developmental stages 22-24 (4.5-day-old) were used. Distal parts of forelimbs and hindlimbs of embryos were isolated and then digested. Cells were counted and resuspended in Ham's F12 medium supplemented with 10% FCS. Mesenchymal cell cultures derived from mouse embryonic limb bud were established according to the aforementioned protocol used by our laboratory on chicken high density cultures with slight modifications for mice. NMRI laboratory mice were mated overnight, the time of detection of the vaginal plug was considered day 0 of gestation. Pregnant female mice were sacrificed by cervical dislocation on day 11.5 of gestation, according to the regulations defined by the University of Debrecen Committee of Animal Research (license number: 11/2010/DE MÁB). The uterus of each animal was removed, E11.5 embryos were then isolated from the uterus, then used to obtain a single cell suspension of chondroprogenitor cells.

Experiments with Inhibitors

To evaluate long term effects of modulating Ca^{2+} homeostasis in chick differentiating chondrocytes, the following compounds were added to the

culture medium of HDC. The L-type VDCC-blocker nifedipine was administered to the culture medium from the beginning of day 1 of culturing at a final concentration of 10 µM. The role of internal Ca^{2+} stores has been ascertained following store depletion with 10 µM of the SERCA inhibitor cyclopiazonic acid (CPA), SOCE was blocked by the simultaneous application of the non-specific TRPC antagonist YM-58483 and $LaCl_3$ (1 µM and 500 µM, respectively). Treatments to address the role of internal stores were performed on culturing day 2 for 24 h. Septin reassembly was inhibited by the addition of forchlorfenuron (FCF) to the culture medium at a final concentration of 200 µM for 24 h on either day 1 or day 2 of culturing.

RT-PCR Analysis

On set days of culturing, HDC established from 100 µL droplets of each model were used for total RNA isolation with TRIzol. Reverse transcription was performed using High Capacity RT kit at 37 °C for 2 h. Specific cDNA sequences were amplified using specific primer pairs designed by Primer Premier 5.0 software based on mouse and chicken nucleotide sequences published in GenBank. The specificity of primer pairs was confirmed *in silico* using the Primer-BLAST service of NCBI before *in vitro* application. Bands representing PCR products were documented with the FluoChem E gel documentation system after horizontal gel electrophoresis in 1.2% agarose gels containing ethidium bromide at 90 V constant voltage for 2 h. Optical density of PCR product signals was determined using ImageJ freeware version 1.46.

Quantitative PCR

The protocol for RNA isolation and reverse transcription was almost identical to what is described in the previous point. Target cDNA sequences were amplified with specific primer pairs designed using the Primer-BLAST service of NCBI. The primers were designed to produce an amplimer sized between 100 and 200 base pairs and span an exon-exon junction, if possible. Relative gene expression levels of chondrogenic and pluripotency marker genes and SOCE-mediating molecules were detected by a QuantStudio 3 Real-Time PCR System in 96-well microplates using 2 × GoTaq® qPCR Master Mix.

For data normalization, the following three reference genes were investigated: *Ppia*, *Rplp0* and *Gapdh*. *Rplp0* was found to show the lowest SD values, therefore it was used to determine relative gene expression levels calculated by the comparative Ct method.

SDS-PAGE, Western Blot and Protein BLAST Analysis

For each sample, 50 µg of protein was separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for immunological detection of investigated molecules. Proteins were transferred electrophoretically to a nitrocellulose membrane. After blocking, membranes were incubated with primary antibodies overnight at 4 °C. Following washing, membranes were incubated with the HRP-conjugated secondary antibody. Membranes were developed by enhanced chemiluminescence reaction and were recorded with the FluoChem E gel documentation system. Optical density of signals was measured using ImageJ 1.46. Protein BLAST analysis of the sequence used for the production of our monoclonal antibody against STIM1 has been performed according to the NCBI BLAST tutorial.

Histological Analysis

For conventional haematoxylin and eosin staining, HDC were fixed with a 4:1 mixture of absolute ethanol and 40% formaldehyde. After rehydration, cultures were stained with Gill's haematoxylin No. 2 and eosin Y. An Olympus DP72 camera on a Nikon Eclipse E800 microscope was used to take photomicrographs of the cultures. Images were acquired using cellSense Entry 1.5 software. The qualitative analysis of cartilage matrix production was performed with dimethyl methylene blue low pH metachromatic staining. For a semi-quantitative approach, we measured the optical density of extracted toluidine blue. Absorbance of these samples was measured at 625 nm with a microplate reader. Matrix mineralization was analyzed by Alizarin Red S staining. Cultures were stained with 2% (w/v) Alizarin Red S dissolved in distilled water for 2 min. Oil Red O staining was applied to estimate lipid accumulation. After fixation, Oil Red O working solution was added for 5 min

at room temperature. Surplus dye was washed away by tap water and then Gill's haematoxylin No. 2 was applied for 20 s.

Determination of Cell Proliferation, Collagen Synthesis and Mitochondrial Activity

To determine the proliferation rate of cells in HDC, ^{3}H-thymidine was added to the wells. Following washing and fixation, plates were dried, then 50 µL scintillation solution was added to each well directly prior to measurements and radioactivity was counted by liquid scintillation counter.

Collagen synthesis was measured very similarly. Simultaneously with 24 h FCF treatments starting either on day 1 or day 2 of culturing, ^{3}H-proline was added to the culturing medium.

MTT-assay was applied to measure mitochondrial dehydrogenase activity immediately after treatments. Cells were incubated for 2 h at 37 °C in MTT-containing Ham's F12 medium. After the removal of the culturing medium, 100 µL of MTT solubilizing solution was added. Absorbance was measured at 570 nm by a microplate reader after 10 min of shaking.

Confocal Microscopy

Line-scan analyses were performed using an LSM 510 META Laser Scanning Confocal Microscope. Cells of 1- and 2-day-old chick HDC were filled with Fluo-4-AM. Imaging was carried out in normal or Ca^{2+}-free Tyrode's solution. Recordings were made with a 63× water immersion objective immediately after adding the test solution to the cultures. Analysis of the images was performed using an automatic event detection software developed by our collaboration partners at the Department of Physiology. X-Y monitoring was done on 1- and 2-day-old Fluo-4-loaded HDC with LIVE 5 Laser Scanning Confocal Microscope using EC Plan-Neofluar 20×/0.50 M27 objective with 2× digital zoom. X-Y image series were recorded after selecting random visual fields. Calcium imaging was performed in normal and Ca^{2+}-free Tyrode's solutions. The rate of frame acquisition was 10 s^{-1}. Zeiss Enhanced Navigation software was used for data analysis.

Single Cell Fluorescent Measurements to Assess Cytosolic Free Ca^{2+} Levels

Measurements were performed using the calcium dependent fluorescent dye Fura-2 on day 2 of culturing. Fura-2-loaded cells were assessed using a 40× oil immersion objective on the stage of an inverted fluorescent microscope. Measurements were carried out in normal and Ca^{2+}-free Tyrode's solutions. SOCE blockers and the agent for store-depletion (CPA) were diluted in Ca^{2+}-free Tyrode's solution. Background fluorescence was subtracted automatically from the signals by the data acquisition software. Cytosolic $[Ca^{2+}]$ was calculated from the ratio of measured fluorescence intensities.

RESULTS

1. Comparative Analysis of Distinct Types of Chondrogenic HDC

Different Morphologies of Primary and Cell Line-Based Micromass Cultures

Unlike in C3H10T1/2-based cultures, nodules were observable in both primary limb bud cultures, while the internodular areas exhibited a low cell density. In accordance with the literature, our results obtained with dimethylmethylene blue (DMMB) staining suggest that chondroprogenitors prefer these densely populated locations to proceed with differentiation into chondroblasts; unlike nodules, internodular areas did not contain metachromatic ECM. On the other hand, no nodules or internodular areas were visible in case of the C3H10T1/2-based cultures.

Examined Models Undergo Various Levels of Chondrogenesis

We have adopted acidic DMMB staining to cultures harvested on selected days of culturing to determine the volume of metachromatic ECM synthesis. The studied models display different temporal arrangements in cartilage ECM accumulation. At day 3 of culturing, we already saw the limb bud-derived HDC embodying large metachromatic territories within their nodules. By the end of the 15-days-long culturing period of both primary HDC, their ECM completely turned metachromatic, its total volume has increased and hypertrophic chondrocytes also appeared to be present. We have also examined mRNA expression profiles of chondrogenic marker genes through the same culturing period. *Sox9*, was detected to be expressed at a constant level in the primary and b-C3H10T1/2 colonies. The same was seen in case of *Col2a1*, but in all examined micromass cultures. mRNA expression of *Acan* was completely absent in c-C3H10T1/2 cultures. *Hapln1* displays a substantial expression in both primary and BMP-2 overexpressing colonies without the signs of ample expression in c-C3H10T1/2 cultures. A marked increase of *Snorc* mRNA amount could be detected from day 3 of culturing in the mouse primary and the BMP-2 overexpressing colonies. *Prg4* has an expression pattern where strong signals are exclusive to post-day 6 mouse limb bud derived HDC.

Primary cultures expressed *Col10a1* in a steady, or slightly increasing fashion, while the cell line-based colonies had low expression levels and an indistinct temporal pattern for this gene.

Assessment of Osteogenic Differentiation

Calcified matrix accumulation was appraised by the application of Alizarin Red staining method. c-C3H10T1/2 cultures did not display signs of calcification, but we could observe a strong positivity in the BMP-2 overexpressing version of the same cell line from day 6 of culturing. Primary micromass cultures, on the other hand, displayed substantial calcification exclusively within their cartilaginous nodules from day 10 (or day 6 in the case of chick HDC). With RT-PCR analysis of osteogenic lineage-specific genes, *Runx2*, evinced a stable expression level in all differentiating HDC, but exhibited a progressive decrease in c-C3H10T1/2 colonies. Expression of *Osx* has a deranged pattern and a relatively weak signal in c-C3H10T1/2 HDC, but shows strong signals from day 3 in the mouse primary and b-C3H10T1/2 colonies. *Col1a1* mRNA expression was substantial and constant in primary and b-C3H10T1/2 colonies. The mRNA expression of late osteogenic markers, *Oc* and *Op*, reveals a disorganized pattern and weak expression in c-C3H10T1/2 HDC. *Ap* was completely undetectable in c-C3H10T1/2 micromass cultures, while a strong upregulation appeared in the other mouse models.

Adipogenic Differentiation

Other differentiation directions also demand consideration in case of mesenchymal cells, in particular, adipogenesis. Oil Red O staining procedure was applied to observe the accumulation of lipid droplets. Haematoxylin was co-applied to tag nuclei. Even by day 15 of culturing, limb bud-derived micromass cultures did not exhibit large lipid droplets, only small ones that are consistent with the normal structure of chondrocytes. The C3H10T1/2 cell line-based cultures, on the other hand, contained cells with Oil Red O-stained droplets of substantial size and number, particularly in the later phase of culturing. Even c-C3H10T1/2 HDC contained numerous adipocyte-like cells; an unexpected result from an apparently non-differentiating model. According to

conventional RT-PCR analysis of adipogenic genes, *Fabp4* mRNA transcripts could be observed in all examined HDC, a marked upregulation was apparent in both the b-C3H10T1/2 colonies and limb bud-derived HDC by late phases of culturing. Nevertheless, *Pparγ2* showed little or no expression in all models except in the late stages of culturing of the b-C3H10T1/2 derived micromass cultures, which is in conformity with the literature regarding adipogenic actions of low BMP-2 concentrations.

Pluripotent Cells in Micromass Cultures

We have evaluated the mRNA expression patterns of key ESC genes – *Nanog*, *Sox2* and *Oct4* (or its avian homologue: *PouV*) – in our models. *Oct4* was not detectable in the mouse HDC samples. Nevertheless, *PouV* appeared distinctly in the avian model, displaying a firmly decreasing tendency towards later stages. However, *Nanog* and *Sox2* were identifiable in all mouse models. *Sox2* was found to be expressed in b-C3H10T1/2 cultures and in both limb bud-derived HDC at moderate levels in the beginning of culturing, but mostly became downregulated as differentiation advanced. Meanwhile, *Nanog* underwent a robust upregulation in mouse differentiating models, especially in the BMP-2 overexpressing HDC, with no detectable expression in the avian colonies. There was no distinct expression pattern for any of the above markers in c-C3H10T1/2 colonies.

2. Investigating Ca^{2+} Signaling Processes in Chick HDC

Cells of Differentiating Chick HDC Display Rapid Spontaneous Ca^{2+} Oscillations

LIVE confocal microscopy was combined with Fluo-4 fluorescent Ca^{2+} imaging technique. The detected oscillations comprise a wide spectrum of amplitude and frequency. On day 1 of culturing, 45 of the 240 studied cells displayed spontaneous Ca^{2+} oscillations, by day 2, this fraction escalated to 55%. Regarding other parameters, the frequency of oscillations manifested a significant decrease by day 2, but the mean amplitude increased significantly. The values of full time at half maximum (FTHM) did not display a significant difference between the two investigated culturing days.

Transformation of Ca^{2+} Oscillations

Next, we have studied the properties of spontaneous Ca^{2+} oscillations in 2-days-old cultures by registering X-Y image series. Every single parameter was normalized to the control value obtained at the respective time point. Treatment with nifedipine did not induce a compelling shift in the percentage of oscillatory cells, or in the amplitude of oscillations, but in turn, it reduced the frequency of Ca^{2+} oscillations. SOCE blockers, on the other hand, caused a significant decline compared to control values at all three time points in the proportion of oscillatory cells and most importantly, no Ca^{2+} transients were observable in any of our random visual fields after 5 min. Furthermore, there was a simultaneous reduction in the amplitudes and the frequency of the Ca^{2+} oscillations. With a higher temporal resolution we saw that the addition of $LaCl_3$ and YM-58483 ceased Ca^{2+} oscillations. The combination of CPA with SOCE blockers led to the prompt abolishment of Ca^{2+} oscillations. Cells displayed a progressive decline in the amplitudes of their oscillations in Ca^{2+}-free Tyrode's.

Components of the Molecular Apparatus of Ca^{2+} Oscillations

Functional VDCCs are composed of a diverse set of subunits, but the Ca^{2+} selective pore-forming α_1 subunit has a principal role in governing the character of the assembled channel. Accordingly, we were able to identify specific bands in the case of certain L- (*$Ca_V1.2$* and *$Ca_V1.3$*), R- (*$Ca_V2.3$*) and T-type (*$Ca_V3.1$*, *$Ca_V3.2$* and *$Ca_V3.3$*) ion channel α_1 subunits and we also identified VDCC α_1 subunit expression at the protein level. We also carried out the characterization of CRAC subunits. A stable expression pattern was seen during the whole culturing period in the case of *Orai1*, *Stim1* and *Stim2* mRNA. In addition, we could also validate the constant protein expression order of STIM1 throughout the complete culturing period with elevated levels at important days of differentiation. Protein BLAST search suggests that in addition to STIM1, STIM2 may also be recognized by the same antibody.

Precise Operation of VDCCs and SOCs is Required for Chondrogenesis

Notably, combined treatments including ER Ca^{2+} store depletion and SOCE blockers that lasted longer than 24 h completely inhibited cartilage formation. Metachromatic staining displayed a seriously abated cartilage matrix production by day 6 with continuous nifedipine treatments. The applied concentration of this pharmacon is approximately one order of magnitude higher than its therapeutic plasma concentration. A similarly noticeable inhibition of chondrogenesis was observed following only 24 hours of ER Ca^{2+} store depletion combined with SOCE blockers on culturing day 2. Nifedipine did not cause any significant alteration in the mRNA expression of the monitored chondrogenic genes, however, we could detect a notable reduction in SOX9 protein expression without considerable decrease in the amount of its phosphorylated form. Combined inhibition of SOCE with ER Ca^{2+} store depletion was found to distinctly diminish ECM component mRNA expression without a similar effect on *Sox9* transcript levels. SOX9 responded similarly at the protein level as in the case of nifedipine treatments. We have also carried out MTT tests and ^{3}H-thymidine incorporation assays on day 3 to measure responses in mitochondrial activity and rate of proliferation, respectively. Notably, none of our tested treatments caused a significant shift in the results of the metabolic activity assay, but almost completely blocked cell proliferation.

Inhibition of Septin Rearrangement

We have also tested the effects of FCF treatment, an inhibitor that blocks the rearrangement of septin filaments. Metachromatically stained colonies displayed definitely decreased cartilage matrix production by day 6 in all cases. Both treatment timings resulted in an increased mitochondrial metabolic activity, but the rate of proliferation displayed a dramatic decrease. According to ^{3}H-proline incorporation assays, collagen synthesis also declined significantly in treated groups. On both days, FCF caused a marked decrease in the expression of all investigated chondrogenic marker mRNAs; all examined markers reached only approximately 20% of their mRNA expression observed

in the solvent control. *Orai1* expression remained almost unaltered following treatment on day 1, but a strong elevation is seen on day 2. At the same time, the investigated pluripotency marker, *PouV*, is substantially upregulated in both cases.

Fluorescent Single Cell Ca^{2+} Measurements

Fluorescent intracellular Ca^{2+} assessments were carried out with Fura-2-loaded colonies on day 2 of culturing to determine the parameters of generated SOCE Ca^{2+} transients without and with the addition of SOCE blockers. Following pre-treatment with the CPA in Ca^{2+}-free Tyrode's, we re-established the normal extracellular Ca^{2+} concentration and recorded the resulting changes in cytosolic Ca^{2+} concentration of individual cells. A significant reduction could be seen in the presence of SOCE blockers in both the amplitude and the maximal rate of rise of the transient when compared to control values.

DISCUSSION

1. Comparative Analysis of Distinct Types of Chondrogenic HDC

Pathways of Mesenchymal Differentiation and Pluripotency

Morphological comparison of examined chondrogenic models revealed that the presence or lack of prechondrogenic nodules was one of the most straightforward differences between the limb bud-derived and the cell line-based HDC. It can be assumed that primary micromass cultures express a relatively high number of intercellular junctions, mainly N-cadherins that provide a more original representation of *in vivo* tissue, where similar nodules also appear. Nevertheless, N-cadherins are also expressed by C3H10T1/2-derived colonies and their expression also influences migratory potential. b-C3H10T1/2 cells displayed a decreased motility when compared to c-C3H10T1/2, which is in accordance with results demonstrating that induction by BMP-2 is necessary for the upregulation of N-cadherin expression. During the investigation of the chondrogenic capacity of involved micromass cultures, we have detected a steady mRNA expression profile for majority of the essential chondrocyte-specific genes in both the primary and the BMP-2 overexpressing HDC. Nevertheless, *Prg4* only demonstrated a strong expression in mouse limb bud-derived colonies (unfortunately, its mRNA sequence was not available for chicken in any of the searched nucleotide databases: NCBI, DDBJ and EMBL-EBI), which is a substantial indication that these cultures have a better coincidence with native articular cartilage. Analysis of metachromatic ECM morphology, arrangement and quality further verified this observation. Our observations suggest all investigated differentiating models undergo notable osteogenic differentiation and matrix calcification. Results regarding the mRNA transcript levels of prominent osteogenic transcription factors, *Runx2* and *Osx*, imply that osteogenesis takes place as soon as the outset of culturing in all but the c-C3H10T1/2 colonies. Previous studies already confirmed the osteogenic potential of both primary micromass and BMP-2 treated C3H10T1/2 micromass colonies. The relatively early onset of osteogenesis is further reinforced by the mRNA expression of

other osteogenic marker genes. Alizarin Red staining to visualize ECM calcification suggests a similarity between the rate and the pathway how these cultures reiterate embryonic endochondral bone formation. Curiously, the expression of articular cartilage markers (such as *Snorc* and *Prg4*), was not separated temporally from bone markers (namely *Oc* and *Op*), suggesting that chondro- and osteogenesis do not follow a sequential pattern as in the case of *in vivo* limb formation. Based on observations in scientific works, we decided to also look for adipogenic signs in all of our investigated models. We were able to detect *Fabp4* mRNAs in all of our micromass models with an apparent upregulation at later stages that was exclusive to the differentiating cultures and was not seen in the case of c-C3H10T1/2 colonies. Our results regarding *Pparγ2* imply that adipocyte differentiation only takes place in late phases of culturing in b-C3H10T1/2 colonies. With a view to this result, we have collected b-C3H10T1/2 HDC for Oil Red O staining on day 25 of culturing where we could observe an immense number of single, large lipid droplet-containing cells (most likely adipocytes) in the colonies. Remarkably, lipid droplet-accumulating cells were also seen in c-C3H10T1/2 cultures, while limb bud-derived colonies contained cells with small and infrequent lipid droplets; these are presumably mature chondrocytes and lipid droplets are present as their natural structural components. In addition, we were also interested in ascertaining whether a portion of the cells in HDC maintain a pluripotent condition. Our results regarding mRNA expression of major pluripotency markers provide an important indication that some cells may actually remain in an undifferentiated status.

Selecting the Right Chondrogenic Model

According to the results seen so far, evidence for the presence of all three examined major mesenchymal differentiation pathways and for cells maintaining a pluripotent state can be seen by both morphological and mRNA expression analyses in the investigated differentiating micromass cultures. Implicitly, cells in these *in vitro* cultures become committed and differentiated towards disparate lineages in a simultaneous manner, instead of a sequential pattern. Moreover, key osteo- and chondrogenic lineage-specific transcription

factor mRNAs are promptly identifiable, while the adipogenic *Pparγ2* expression can only be observed in the second half of culturing and exclusively in b-C3H10T1/2 colonies. On the other hand, an upregulation of marker gene transcripts for all three lineages was noticed by day 15 in primary mouse limb bud HDC. In primary chicken limb bud HDC, similar tendencies can be inferred. b-C3H10T1/2 HDC demonstrate the most convincing signs of adipogenesis, while our observations in both limb bud-derived micromass colonies likely indicate lipid droplets that are natural constituents of mature chondrocytes. Our data provides a solid confirmation to support that the embryonic limb bud-based models produce a closer recapitulation of embryonic cartilage formation and endochondral ossification. Among limb bud-based systems, the avian model offers an immense convenience due to its simplicity, reproducibility, high yield and cost-effectiveness; unless some of the known disadvantages (converse regulation of certain pathways, lack of available antibodies or sequences, inefficiency of particular drugs) compromise the experiments or the interpretability of gathered data, these advantages easily outweigh other factors.

2. Ca^{2+} Homeostasis in Differentiating Chondrocytes

VDCCs in Chondrogenic Cells

Ca^{2+} is broadly acknowledged as the most adaptable second messenger. There is growing data to indicate that non-excitable cells, such as MSCs and chondrocytes, are utilizing Ca^{2+} signaling pathways as pivotal mediators throughout the course of differentiation. As L-type VDCC-specific blockers nifedipine and verapamil abated cartilage differentiation in mouse limb bud-derived micromass cultures, the significance of these plasma membrane Ca^{2+} channels is already established in this model. Previous results of our laboratory are in agreement with the above. The preceding set of data clearly indicates the importance of Ca^{2+} dependent signaling, more specifically, extracellular Ca^{2+} as a source of influx via plasma membrane Ca^{2+} channels in chondrogenesis.

Accordingly, we have completed a comprehensive characterization of Ca_V α_1 subunit mRNA expression to be able to identify detectable transcript levels in the case 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. Moreover, we have found an indication for the role of VDCCs throughout chondrogenic differentiation based on the continuous presence of Ca_V α_1 subunit proteins over the complete culturing period and results from the continuous application of nifedipine from day 1. Both cartilage-specific ECM production and SOX9 protein expression were markedly reduced by the treatments. T-type VDCCs are particularly likely to play a major role in regulating the cell cycle of non-excitable cells due to their favorable characteristics. Remarkably, all three T-type channels are constantly expressed in chicken HDC, which is a definite indication of their great influence. As high cellular density is a highlighted requirement of *in vitro* chondrogenesis, it was expectable that restricting cellular proliferation by blocking VDCCs would impede chondrogenic differentiation of HDC. Indeed, nifedipine demonstrated the anticipated antiproliferative effects as determined by our proliferation assays. Consequently, we conclude that VDCCs are likely influential regulators of cell division; which has been previously emphasized as a prerequisite to the condensation phase in mesenchymal chondrogenesis.

SOCE in Differentiating Chondrocytes

Another possible source of cytosolic Ca^{2+} elevation is Ca^{2+} release from ER Ca^{2+} stores via either InsP3R or RyR channels. InsP3R mRNAs and proteins was detectable in our model, while no functional RyR expression was identifiable. Signaling pathways leading to InsP3 production may easily deplete the ER Ca^{2+} store of cells of chicken limb bud cultures, since these stores are relatively small in non-excitable cells. As a consequence of store depletion, CRACs become activated to refill the ER stores, SOCE is thus implemented. Notably, detailed characterization of CRACs and other SOCE molecules in mature or developing chondrocytes has not been performed previously to this work. The mRNA expression levels of *Orai1*, *Stim1* and *Stim2* all turned out to be stable during the whole extent of culturing. Furthermore, we were also able

to validate the expression of STIM1 at the protein level. Over and above, in our experiments, application of CPA in combination with SOCE blockade caused the almost complete abolishment of *in vitro* cartilage formation; this implies that ER stores and SOCE appear to play a critical role in the normal Ca^{2+} homeostasis of differentiating chondrocytes. Highly similar results were observed after targeting SOCE via an alternative mechanism, by inhibiting the reorganization of septin filaments, which is a recently described requirement of CRAC activation. After Ca^{2+} homeostasis was disturbed as a consequence of deficient replenishment of ER Ca^{2+} stores, decreased cell division emerged as one of the likely factors behind abated chondrogenesis. Actually, the proliferation rate both after SOCE-block combined with ER Ca^{2+} store depletion or the inhibition of septin remodeling dropped to very low levels, while we have detected little to no effect on the mitochondrial metabolic activity. It is also noteworthy that PouV expression responds in a differentiation stage-dependent manner to disturbances of SOCE, which suggests that the role of Ca^{2+} homeostasis reaches beyond maintaining the rate of proliferation. Also, the highly elevated mRNA expression of *Orai1* after FCF treatment during the period of differentiation (day 2) proposes a compensatory mechanism and an important role played by functional CRACs in the process of differentiation, not only proliferation.

SUMMARY

This work summarizes the results of our research regarding the differentiation of the C3H10T1/2 cell line and primary limb bud-derived micromass cultures in the trilineage mesenchymal direction, as well as the Ca^{2+} homeostasis of embryonic chicken chondrogenic limb bud HDC. The most important results and conclusions of this PhD thesis are as follows:

- Our comparative study demonstrated a largely punctual correlation between morphological and molecular investigations. Based on our analysis, limb bud cultures are apparently a more suitable alternative to model osteochondral differentiation *in vitro* than the examined cell line; chondrogenic nodule formation and the more robust expression of relevant marker genes combined with the apparent absence of adipogenesis suggests that cartilage of a higher quality is formed.
- Morphological signs of adipogenesis were mainly found in c- and b-C3H10T1/2 micromass cultures, while the BMP-2 overexpressing cells also demonstrated the upregulation of *Pparγ2*, which is an adipocyte-specific nuclear hormone receptor and a key regulator of adipocyte differentiation.
- Although mRNA levels of the pluripotency markers display a relatively ambiguous pattern, the orderly decreasing tendency in the expression of the avian homolog of *Oct4*, *PouV* clearly suggests the proper advancement of differentiation. A gradually increasing number of cells lose their pluripotency in chicken limb bud HDC, but pluripotent cells probably remain present in the cell cultures until the end of the investigated period.
- We were able to observe rapid Ca^{2+} transients displayed by cells of differentiating chicken HDC on days 1 and 2 of culturing and we have noted that the percentage of oscillatory cells demonstrated significant modifications concurrently with the progress of chondrogenesis.

- In addition to previously described contributing factors, we have detected the expression of essential components of the molecular machinery needed to generate and maintain oscillations: mRNAs of α1 subunit of VDCCs (L-, R-, and T-type) and SOCE orchestrators (*Stim1*/*Stim2* and *Orai1*); protein level expression of the Ca^{2+} selective pore-forming α1 subunit of VDCCs and STIM1/STIM2.
- We have shown that SOCE blockers impair the parameters of SOCE induced by store depletion during fluorescent single cell Ca^{2+} measurements of chicken limb bud HDC.
- It appears that Ca^{2+} entry blockers are able to cause serious disturbances in the Ca^{2+} homeostasis of cells of chick micromass cultures, while the previous treatment combined with store depletion had even more severe effects, suggesting the importance of both the stores and the extracellular milieu in the maintenance of Ca^{2+} oscillations; blockade of L-type Ca^{2+} channels only appeared to influence the frequency of Ca^{2+} transients; oscillating cells displayed a progressive decline in the amplitudes of their transients in Ca^{2+}-free Tyrode's solution. The latter – in addition to further underlining the role of extracellular Ca^{2+} – hints at the function of extrusion mechanisms, which have not been investigated in this work.

Functionally, both blockade of L-type Ca^{2+} channels and SOCE inhibition combined with store depletion resulted in decreased amounts of metachromatic ECM by the end of the culturing period. However, only interference with SOCE caused the diminishing of expression of key chondrogenic marker genes at the mRNA level; the change in protein levels displays a more unclear picture, but regardless, the amount of the active P-SOS9 form also declined in response to SOCE inhibition, but not the blockade of L-type VDCCs. The rate of proliferation decreased dramatically in the case of both treatments without a significant drop in mitochondrial activity ('viability'), further underlining the essential role of Ca^{2+} signaling in the cell cycle.

Our results suggest that various elements of Ca^{2+} homeostasis may have different depth to their impact while exerting influence on a particular cellular function or molecular pathway, i.e. SOCE rather than VDCCs influences mRNA expression of cartilage ECM specific molecules, while proliferation of cells appears to be sensitive to any disturbance in the physiological pattern of cytosolic [Ca^{2+}] changes.

PUBLICATIONS

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List of publications related to the dissertation

1. **Takács, R. Á.**, Matta, C., Somogyi, C., Juhász, T., Zákány, R.: Comparative Analysis of Osteogenic/Chondrogenic Differentiation Potential in Primary Limb Bud-Derived and C3H10T1/2 Cell Line-Based Mouse Micromass Cultures.
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