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

Investigating the regulatory mechanisms of DNA methylation and circadian clockwork during chondrogenesis

by Judit Vágó

Supervisor: Róza Zákány, MD, PhD

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Supervisor: Róza Zákány, MD, PhD

Doctoral School of Molecular Medicine, University of Debrecen

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Introduction

Musculoskeletal disorders are among the most prevalent health problems of the world's society. Severe physical impairments may lead to chronic pathological conditions such as osteoarthritis (OA), which is associated with irreversible degeneration of articular cartilage, inflammation and intense pain within the joint cavity. A study published in 2019 stated that approximately 1.71 billion people of the world's population have abnormal musculoskeletal conditions, of which the number of people suffering from OA can reach 343 million. Sedentary lifestyle, neglect of physical activity and unhealthy, calorie-rich nutrition leading to obesity are the most common causes of the disease. These characteristic lifestyle problems affect more and more people even at younger ages, especially in developed countries. Prolongation of life expectancy and population ageing are also major risk factors. Based on these circumstances, scientists predict that joint disorders will affect an increasing number of people in the future. There is no effective treatment for OA and the therapeutic medication for cartilage regeneration is very limited. The current approach is restricted to symptomatic treatment with anti-inflammatory analgesics, and eventually total joint replacement. One potential treatment for damaged hyaline cartilage and for future therapeutic applications of cartilage tissue engineering is the chondrogenic differentiation and implantation of mesenchymal stem cells. Knowledge of the regulatory pathways and factors involved in the differentiation mechanisms of these mesenchymal cells is limited. To establish proper therapeutic procedures and understand the fundamental biological characteristics of articular cartilage development, basic research of the chondrogenic differentiation of mesenchymal cells is essential. The Signal Transduction Research Group in the Department of Anatomy, Histology and Embryology of the University of Debrecen has an area of interest in cartilage biology. Our aim is to find out what molecular and biochemical changes accompany the healthy and pathological differentiation of cells during cartilage formation. Our results may contribute to the development of novel therapies which may help restoring the original ultrastructure of degenerated hyaline cartilage.

In vitro cartilage formation (chondrogenesis) consists of three characteristic differentiation stages. Chondroprogenitor cells are isolated from the developing limb buds of chicken or mouse embryos and inoculated into plastic tissue culture dishes at high concentration (e.g. 1.0×10^7 or 1.5×10^7 cells/mL) to establish *high density* (HD) or *micromass* primary chondrifying cultures. During the first phase, proliferation and migration of cells occur between culturing days 0 and 3. Chondroprogenitor cells and early chondroblasts are characteristically present in the micromass culture, which create cell aggregations or nodules. The next phase of

differentiation takes place between culturing days 3 and 6. Micromass cultures are mainly built up by differentiating chondroblasts and early chondrocytes that produce high amount of cartilage-specific extracellular matrix (ECM). By day 6, a distinguishable amount of metachromatic ECM is synthetized by chondrocytes. After culturing day 6, mature chondrocytes transform into hypertrophic chondrocytes, which will lead to intense calcification of the micromass culture. Biological processes of hyaline cartilage development and endochondral ossification can be studied with the help of the *in vitro* chondrifying micromass cultures. We can identify many chondrogenic markers that play a crucial role during chondrogenesis, such as SOX9, which is the key transcription factor of cartilage formation, and it is necessary to regulate the expression of cartilage-specific ECM genes such as *COL2A1* (collagen type II alpha 1 chain) and *ACAN* (aggrecan). Examining the expression patterns of these genes after treatment with different compounds may indicate specific molecular changes during chondrogenesis.

The experiments demonstrated in my thesis are based on two relatively new and currently trending scientific areas. Our aim was to study the role of epigenetic and circadian clock-related regulatory pathways during *in vitro* cartilage formation.

Epigenetics refers to reversible and heritable biological mechanisms that regulate gene expression without the modulation of the primary DNA sequence. DNA methylation causes hypermethylation of designated genomic regions and leads to transcriptional inactivation or silencing. This reversible process is catalysed by DNA methyltransferases. DNA demethylation is induced by TET enzymes, and the protein called OGT has a specific role in governing the biological activity of TET enzymes. The balance between DNMT3A and TET1/OGT enzymes defines the actual methylation status of the genome, thus it may influence the spatiotemporal expression of genes involved in cartilage formation.

Biological processes go through cyclic changes during the day in living organisms. This periodic, approximately 24 hours long regulatory mechanism is called circadian rhythm. The mammalian molecular circadian clockwork is regulated by a number of oscillator elements. The main regulatory system is found in the autonomous clock of the central nervous system, which interacts with the peripheral clocks and sends signals of rhythmicity to the nucleus of every cell of peripheral tissues. The central oscillators of the molecular biological clock are coordinated by the transcriptional-translational feedback loop, which means that certain proteins inhibit their own mRNA production after reaching a specific concentration in the cell. We can differentiate between positive (BMAL1, CLOCK) and negative (PERIOD,

CRYPTOCHROME) feedback factors that create a close interconnection with each other. The molecular components of the circadian clockwork affect the expression of several target genes, which means that the tissue-specific clocks on the periphery might have specific biological functions. Knowledge is limited about the adaptation and function of molecular clocks situated in cells found in peripheral tissues, specifically, in the local microenvironment of cartilage.

The principal aim of my work was to present a detailed analysis of the molecular mechanisms of DNA methylation and circadian clockwork which regulate *in vitro* chondrogenesis, and identify new methylation- or clock-specific regulatory molecules that might have an important role in chondrocyte differentiation and cartilage development. These factors may serve as promising therapeutic targets for several human joint disorders, including OA.

Aims and objectives

Our aim was to address the following questions using primary chondrifying micromass cultures established from chicken or mouse embryonic limb buds, cell line-based chondrifying micromass cultures or cryosections of whole mouse embryos:

- **Is it possible to detect the expression of DNA methylation regulatory factors at the mRNA level, and if so, how do they change during the process of *in vitro* chondrogenesis?** At which developmental stage can we observe the transcriptional maximum of the genes? Are there any differences in the gene expression levels and transcriptional activities between the experimental models used for these studies?
- **Does DNA methylation inhibition cause any morphological and molecular alterations in differentiating chondrocytes?** Is there any difference in the effects of inhibition if it is applied during the early or the late phase of *in vitro* cartilage formation? How does the methylation pattern of differentiating or mature chondrocytes change after inhibiting DNA methylation?
- **Is it possible to detect the expression of circadian clock regulatory factors at the mRNA level, and if so, how do they change during the process of *in vitro* chondrogenesis?**
- **Is it possible to synchronize the circadian clock of differentiating chondrocytes?** Can we detect the periodic fluctuation of clock- and cartilage-specific genes, which is a characteristic sign of the rhythmic circadian function? What are the typical features of the relative oscillatory patterns of the examined genes? Is there any difference in the rhythmic circadian expression pattern of the examined genes if synchronization is applied during the early or the late stage of *in vitro* chondrogenesis?
- **Does circadian clock inhibition cause any morphological and molecular alterations in differentiating chondrocytes?**

Materials and methods

Experimental models

High density (HD) or micromass cultures are used for studying *in vitro* chondrogenesis. Some of the primary HD cultures were established from mouse limb bud-derived chondroprogenitor cells derived from 11.5-day-old mouse embryos. Pregnant female mice were sacrificed on a designated day according to the ethical standards defined by the University of Debrecen Committee of Animal Research (Permission No. 2/2018/DE MÁB). Distal parts of fore and hind limb buds were removed from the 11.5-day-old embryos, then limb buds were dissociated in 0.25% trypsin and dissociation was stopped by the addition of foetal bovine serum (FBS). After several centrifugation and filtering, cells were resuspended in high glucose DMEM medium at a concentration of 1.0×10^7 cells/mL. Cell cultures were maintained in a CO_2 incubator at 37 °C (5% CO_2, 90% humidity). Day of inoculation was considered as day 0 of culturing.

Chicken embryos were also used for establishing primary high density cultures. Research studies with early stage chicken embryos does not require the permission of the Ethics Committee of the University of Debrecen. Mesenchymal cells were isolated from limb buds of chicken embryos of Hamburger-Hamilton stages 22 to 24 (4.5-day-old) for the establishment of micromass cultures. Preparation of the limb buds and isolation of the chondroprogenitor cells differed in just a few steps compared to HD cultures with mouse embryo limb bud-origin. In this case, the concentration was adjusted to 1.5×10^7 cells/mL. Cells were maintained in Ham's culture medium supplemented with 10% FBS.

C3H10T1/2 cell line was also used for the study of *in vitro* chondrogenesis. The BMP-2 overexpressing pluripotent C3H10T1/2 cell line originating from mouse embryonic fibroblasts was a gift from our collaboration partner (G. Gross). Cells were first cultured in monolayer system, and after reaching approximately 80% confluence, cells were detached from the surface of the tissue culture dish by trypsinisation. Finally, HD cultures were established by setting the cell density at 1.0×10^7 cells/mL.

In situ hybridization

We applied a nonradioactive, indirect labelling with digoxigenin (DIG) *in situ* hybridization protocol for our experiments. First, we prepared the DIG-labelled RNA probes. We designed specific PCR primers that amplify a ~1000 bps long region from the 3'UTR of *Dnmt3a*, *Ogt* and *Tet1* genes. We applied insert flanking T7 promoters for generating antisense probes. The gene products of *Dnmt3a*, *Ogt* and *Tet1* probes were amplified with the help of PCR from

plasmids. The Roche High Pure PCR Product Purification Kit was used for the isolation of amplified PCR products. RNA labelling was created with a DIG-RNA labelling mix by *in vitro* DNA transcription. The labelled RNA pellet was precipitated from our samples and dissolved in hybridization buffer. We used sections of 15-day-old mouse embryos for the *in situ* hybridization reactions. Pregnant female mice were sacrificed on gestational day 15. After removing the embryos, specimen were fixed in 4% paraformaldehyde overnight, then the embryos were placed in a cryostat and 20 μm thick frozen sections were cut in a sagittal plane. After drying the samples, sections were treated with 100 μL Proteinase K solution (20 μg/mL). Following prehybridisation, slides were incubated for 16 hours with hybridisation solution containing the RNA probe (1-2 μg/mL). Until this step, all substances had to be RNAse free. Samples were washed with saline sodium citrate solution several times, then they were treated with RNAse A solution (0.5 μg/mL). Slides were incubated overnight with 10% Blocking buffer solution containing α-DIG antibody (1:1000). The proper reaction of DIG antibody was detected with TRIS-NBT/BCIP solution at room temperature in the dark for 2~20 hours. At the end of the incubation, sections were mounted with DPX medium and digitized using an Olympus BX53 camera on a Nikon Eclipse E800 microscope. Photomicrographs were analysed with ImageJ.

PCR array

Quantitative PCR arrays were used to verify the expression of 80 epigenetic-, chondrogenic- and osteogenic-associated marker genes. For this experiment, we used micromass cultures established from C3H10T1/2+BMP-2 cells and cultures were harvested according to the specific states of *in vitro* chondrogenesis. Direct-Zol® RNA Miniprep kit was used for total RNA isolation. cDNA synthesis of the samples containing 1000 ng total RNA was conducted by iScript RT Supermix kit. Specific primers were designed with PrimerQuest software. RT-qPCR was carried out in a CFX RT-PCR machine using SsoFastEvaGreen™ Supermix. Parameters of the PCR reactions were set based on the manufacturer's instructions. Heatmap-based analysis of the results was conducted by CFX Maestro software.

Total RNA isolation and reverse transcription

First, micromass cultures were washed on specific culturing days with physiological NaCl two times and stored at –80 °C. In case of chicken embryonic limb bud-derived HD cultures, RNeasy kit was used for total RNA isolation, according to the manufacturer's protocol. Total RNA isolation from primary mouse embryonic limb bud-derived HD cultures and some micromass cultures established from C3H10T1/2+BMP-2 cells was trizol-based which means

that cultures were mixed with TRI Reagent. After the addition of 20% RNAse-free chloroform, samples were centrifuged and incubated in 500 µL RNAse-free isopropanol.The isolated total RNA was dissolved in RNAse-free water and stored at –80 °C. RNA concentration and purity were determined by NanoDrop 1000 spectrophotometer. Reverse transcription reactions were performed on 1 µg of total RNA using High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol.

Quantitative real-time PCR analysis

The mRNA expression patterns of specific marker genes were examined by SYBR Green fluorescent dye-based RT-qPCR reactions. Specific primer pairs were designed by the Primer-BLAST service of NCBI. First, conventional PCR was carried out with Promega GoTaq Flexi DNA Polymerase kit to generate standard curves for absolute quantification. Amplified PCR product purification was conducted with Roche High Pure PCR Product Purification Kit according to the manufacturer's instructions. Standard curves were established by diluting purified PCR products in a serial manner (10-fold, starting with 1 ng/µL). QuantStudio 3 Real-Time PCR System, GoTaq qPCR Master Mix and 10 ng cDNA per each 10-µL reaction was used for the RT-qPCR reactions. Amplification data were examined using the QuantStudio Design and Analysis Software and Microsoft Excel. We had to choose an optimal reference gene in order to determine biological differences in our samples. The suitable normalizing gene was selected using the NormFinder algorithm.

Quantitative methylation-specific PCR analysis

DNA methylation was examined by quantitative methylation-specific PCR. EZ DNA methylation-direct™ kit was applied for genomic DNA purification and bisulfite conversion of the template based on the company's manual. Primers were designed using MethPrimer 2.0 software. TATA box binding protein (*Tbp)* promoter-specific unmethylated primer were used for normalization of qPCR data sets, it served as a negative control. qMSP reactions were carried out in a CFX96 PCR machine, data sets were analysed with CFX manager software.

Synchronizing the circadian clock

Synchronizing the expression pattern of circadian clock-specific genes was achieved by serum shock in chicken embryo limb bud-derived HD cultures which was applied on the first or the sixth day of culturing. During serum shock, normal F12 culturing medium containing 10% FBS was replaced with F12 medium containing 50% FBS for two hours. After removing the serum-rich medium, normal F12 (containing 10% FBS) was added to the chondrifying colonies. Control cultures received fresh F12 medium (containing 10% FBS) during serum-shock. Time

point of synchronization was considered as the starting point of the experiment (time point 0). Samples were harvested every 8 hours between 24 and 72 hours postsynchronization. Some cultures were collected on the third or sixth day of culturing after synchronization on the first culturing day, thus we could examine the long term effects of synchronization (and also the effects of a circadian clock modifier called Longdaysin) on *in vitro* cartilage formation.

Pharmacological modulation of *in vitro* chondrogenesis

5-azacytidine (5-azaC) was used for investigating the role of epigenetic regulation during *in vitro* chondrogenesis. The compound inhibits the connection of DNA methyltransferases to their specific binding sites, thereby prompting DNA demethylation and activating specific gene regions. 5-azaC was dissolved in dimethyl-sulfoxide and it was applied in a final concentration of 10 μM on culturing days 1 or 3 for 72 hours on mouse embryo limb bud-derived micromass cultures. Samples were collected on culturing day 4 or 6. The molecular clockwork was modified with a chemical agent called longdaysin (LDS). It inhibits the activity of casein kinase I which has a role in the phosphorylation of PERIOD proteins. Without phosphorylation, PERIOD proteins are not eliminated through proteasomal degradation and the clockwork is dysregulated. LDS was dissolved in dimethyl-sulfoxide and it was applied in a final concentration of 5 μM in chicken embryo limb bud-derived HD cultures on culturing day 1 (after serum shock) for 24 hours. Control cultures received the same treatment protocol but with dimethyl-sulfoxide. Samples were collected on culturing day 3 or 6.

Mitochondrial activity

MTT reagent (5 mg/mL) was added on specific culturing days to the medium of samples. At the end of incubation, the medium was removed and MTT solubilizing solution was added. Optical density of the liquid containing the extracted MTT reagent was measured at 570 nm with a microplate reader.

Cell proliferation assay

1 μCi/mL ^{3}H-thymidine (185 GBq/mM) was added to the medium of mouse embryo limb bud-derived HD cultures on designated culturing days. Cultures were incubated for 16 hours, then proteins were precipitated with ice-cold 5% trichloroacetic acid. Samples were air-dried for one week. Finally, scintillation liquid was pipetted onto the cultures and radioactivity was measured by a liquid scintillation counter.

Metachromatic staining methods

As for dimethyl-methylene blue (DMMB) staining method, cultures were fixed in 4:1 mixture of absolute ethanol and 40% formaldehyde, then rehydrated in a descending series of alcohol,

and cultures were stained with 0.1% (*w/v*) DMMB dissolved in 3% (*v/v*) acetic acid. Finally, cultures were mounted with Aquatex mounting medium. Cryosections of whole 15-day-old embryos were also stained with DMMB. First, frozen sections were dried, then samples were stained with 0.1% (*w/v*) DMMB dissolved in distilled water. Finally, slides were mounted with DPX. Photomicrographs of the samples were taken using an Olympus BX53 camera on a Nikon Eclipse E800 microscope. As for toluidine blue (TB) staining, cultures were fixed with Kahle-fixative, then stained with 0.1% toluidine blue dissolved in glycine–HCl buffer. The dye bound to the negatively charged cartilage matrix macromolecules was extracted in 8% HCl dissolved in absolute ethanol. Absorbance of samples was measured at 625 nm by a microplate reader.

Statistical analysis

Data in figures is representative of at least three independent experiments, averages are expressed as mean ± SEM (standard error of the mean) or SD (standard deviation). numerical data was analysed using Microsoft Excel. Rhythmicity of circadian clock marker genes was defined by fitting expression data with a nonlinear cosinor regression. Parameters of the standard cosine function was examined using Prism. Statistical analysis was performed using paired Student's *t*-test or One-Way ANOVA with Tukey HSD and Mann-Whitney test. Specific differences were considered statistically significant if $P < 0.05$.

Results

Investigation of DNA methylation regulatory mechanisms during *in vitro* and *in vivo* chondrogenesis in mouse embryonic models

First, we established chondrogenic HD cultures from C3H10T1/2+BMP-2 cells for DNA methylation-oriented experiments. Micromass cultures were harvested on designated days of culturing based on the specific phases of *in vitro* chondrogenesis. We investigated the expression patterns of three DNA methylation-associated genes (*Dnmt3a, Tet1, Ogt*). Based on the results of PCR array, *Dnmt3a* expression peaked on culturing day 15, *Tet1* was strongly expressed on day 10, and *Ogt* showed upregulation between day 10 and 15. The expression of cartilage-specific ECM marker genes *Col2a1* and *Acan* displayed upregulation during the early stage of chondrogenesis (between day 5 and 10), while the hypertrophic chondrocyte-specific *Col10a1* or the osteogenic differentiation-specific *Col1a1* was strongly expressed during later stages (between day 10 and 15).

We studied the relative expression patterns of the three DNA methylation marker genes using quantitative PCR. Micromass cultures established from C3H10T1/2+BMP-2 cells were collected on specific culturing days. The most prominent expressional changes were examined on culturing day 10 for all three genes, this was in good correlation with the results of the PCR array. The transcript level of *Tet1* showed the largest changes: it was first upregulated from culturing day 5, and its expression level was still significantly elevated on culturing day 15.

We continued our research with the analysation of *Dnmt3a*, *Tet1*, and *Ogt* genes in primary HD cultures established from mouse embryonic limb buds. Samples were collected on specific culturing days according to the developmental stages of *in vitro* chondrogenesis and expression patterns were examined using quantitative PCR. *Tet1* again displayed the highest expressional shift of the three genes, its transcription was significant on every culturing day, with peaks on days 1 and 4. Transcript level of *Dnmt3a* was significantly high on culturing day 3, and it was downregulated by day 15. In case of *Ogt*, we examined a constant expression level of the gene in the differentiating chondrocytes, but it was also downregulated by day 15.

We also intended to demonstrate the *in vivo* relevance of the three epigenetic marker genes in chondrogenesis, thus we analyzed 15-day-old whole mouse embryo cryosections using *in situ* hybridization to examine the expression of *Dnmt3a*, *Tet1* and *Ogt* at mRNA level. The signal intensities of the applied specific RNA probes were different: *Ogt* showed a weak expression

pattern, *Dnmt3a* appeared to have a moderate expression level, while *Tet1* displayed the strongest expression signals in the primitive limb and vertebrae.

Our aim was to study the importance of the regulatory pathways of DNA methylation during *in vitro* chondrogenesis. For this reason, we applied the inhibitory agent 5-azaC at a final concentration of 10 μM on chondrifying HD cultures established from mouse embryonic limb buds. Two treatments protocols were created: some HD cultures were treated with 5-azaC between culturing days 1 and 4, while other cultures were from culturing day 3 to day 6. With this method, we could examine the effects of inhibiting DNA methylation (thus causing demethylation) on early or late phase of *in vitro* cartilage formation. The amount of the produced cartilage specific ECM was significantly lower for both inhibitory protocols. The cell proliferation ability was also negatively affected in both cases. The early stage of chondrocyte differentiation was more strongly altered by the treatment than the late stage of chondrogenesis. We also examined the cytotoxic ability of the treatment: differentiating cells in 4-day-old primary HD cultures showed a significant reduction, but the 6-day-old cultures demonstrated an immense loss in their viability. We also studied the expression patterns of the three DNA methylation-associated marker genes (*Dnmt3a*, *Tet1*, *Ogt*) and three cartilage marker genes (*Sox9*, *Col2a1*, *Acan*) using RT-qPCR. We verified that the modulation with 5-azaC significantly downregulated the expression of *Dnmt3a* in both cases of treatment protocols. The cartilage specific genes showed a significant downregulation when the treatment was applied during the early phase of chondrogenesis. *Col2a1* and *Acan* displayed the largest decrease of expression. In contrast, 5-azaC applied during the late phase of chondrogenesis caused a significant upregulation of *Sox9* and *Acan*. The effect of 5-azaC on the methylation status of genes was studied by quantitative methylation-specific PCR. The treatment did not affect the methylation status of the promoter regions of cartilage specific genes during early phase of chondrocyte differentiation, but it caused a significant decrease in the methylation level of *Sox9* and *Acan* promoter regions. These data were in a good correlation with the RT-qPCR results, thus we could conclude that the elevation of *Sox9* and *Acan* gene expression levels in 6-day-old primary HD cultures were direct consequences of 5-azaC treatment.

Investigation of circadian clock regulatory mechanisms during *in vitro* chondrogenesis in chicken embryonic models

To examine the molecular changes of the daily (approx. 24 hours long) circadian rhythm, we had to synchronize the chondroprogenitor cells of the applied *in vitro* chondrifying model

system. Our primary aim was to study the role of the circadian clockwork at a time point during the early stage of *in vitro* cartilage formation, when differentiation of chondroblasts and ECM synthesis is the most intense. Based on the results of specific histological staining methods we decided that the optimal time point for synchronization was culturing day 1, and the first sample had to be collected on the second day of culturing (24 hours post synchronization). We also synchronized the cells of 6-day-old HD cultures, and the first sample was collected on culturing day 7 (24 hours post synchronization), which made it possible to examine the role of molecular circadian clock during later stages of chondrogenesis.

Chicken embryonic limb bud-derived micromass cultures were synchronized on the first culturing day with serum shock, which means that cultures were stimulated with culturing medium containing 50% FBS. Cartilage specific ECM production was studied using DMMB and toluidine blue histological staining methods. When cultures were exposed to serum shock, the synthetized metachromatic ECM was significantly higher from culturing day 3, the largest increase was detected on the 4th culturing day. These results suggest that serum shock applied during the early stage of *in vitro* chondrogenesis significantly stimulates cartilage formation.

We wanted to prove that the elevated level of ECM production was a consequence of circadian clock synchronization caused by serum shock. LDS can modulate the function of circadian clock. The chemical agent was applied after the serum shock on the first day of culturing for 24 hours in the HD cultures. The treatment significantly reduced the amount of cartilage specific ECM by culturing day 6, and it also altered the metachromatic matrix production of serum-shocked cultures in a negative way. We verified with histological procedures that the increased chondrogenic differentiation was a direct consequence of circadian clock synchronization induced by serum shock. Mitochondrial activity was affected by the inhibitory factor in a negative way: the treatment led to a significant decrease in cell viability by culturing day 3.

We investigated the effect of LDS on the molecular mechanisms of chondrogenesis. We examined the expression pattern of three chondrogenic marker genes (*SOX9*, *COL2A1*, *ACAN*) with RT-qPCR. After synchronization and/or the treatment with LDS, primary HD cultures were harvested on culturing day 3 or 6. The relative expression of the three marker genes was significantly high in serum-shocked cultures on both culturing days. After serum shock and treatment with LDS, however, the expression levels of the examined genes significantly decreased compared to serum-shocked cultures. All in all, we concluded that the LDS-treatment had a negative effect on *in vitro* chondrogenesis and on synchronization by serum shock. Based on our results we declared that the proper function of the molecular clock is required for cartilage formation. These studies also prove that the positive effect of serum shock applied

during *in vitro* chondrogenesis can be significantly decreased by pharmacological inhibition of the molecular clockwork in differentiating chondrocytes.

We aimed to prove that serum shock had a synchronizing effect on the molecular regulatory elements of the circadian clock and chondrogenesis. Chicken embryonic limb bud-derived micromass cultures were synchronized with serum shock on culturing day 1 or 6. Samples were collected every 8 hours between 24 and 72 post synchronization. Expression patterns of the examined genes and their oscillation peaks (which marks the circadian rhythmic function) were analyzed by RT-qPCR. We verified the expression of clock-specific genes *BMAL1*, *PER2*, *PER3*, *CRY1* and *CRY2* in our chicken embryo limb bud-derived chondrogenic model, but we were unable to detect transcripts for *CLOCK* genes. We identified the synchronized oscillatory expression pattern of many clock-specific genes in HD cultures that were serum-shocked on culturing day 1. We discovered an approximately 8-hour phase shift between the positive phase and negative phase of the clock over the 72 hours investigated time period after serum shock. The gene expression data was fitted with a nonlinear cosinor regression. Sinusoid patterns of cosine fits showed *BMAL1* as positive, and *PER2*, *PER3*, *CRY2* as negative factors of the circadian rhythm. We observed the same gene expression patterns when HD cultures were synchronized on culturing day 6. *BMAL1* and *CRY1*/*PER2* genes showed an 8-hour phase angle difference which referred to a functional molecular clock in this experimental group as well.

Progenitor cells in the chicken embryonic limb bud-derived micromass cultures represent a heterogeneous population where cells may preferentially choose osteogenic differentiation instead of chondrogenic differentiation. To clarify this situation, we examined the gene expression patterns of the key chondrogenic (*SOX9*) and osteogenic (*RUNX2*) transcription factors at the previously described time points. Gene expression values were fitted with the cosine function to study the oscillatory patterns specific for circadian clockwork. Expression of *SOX9* gene showed a circadian rhythmic pattern during both early and late phases of chondrogenesis and it was largely in phase with *BMAL1* oscillation. In contrast, no circadian oscillatory pattern could be identified for *RUNX2* expression. The cosinor-based analyzation of *ACAN* gene expression showed a similar circadian rhythmic pattern which was previously observed for *SOX9* in HD cultures synchronized on both days 1 and 6. *COL2A1* displayed a circadian trend similar to those of *SOX9* and *ACAN* in both immature chondroprogenitor cells and mature chondrocytes. The relative gene expression results suggest that chondrogenic marker genes are regulated by the molecular circadian clock in HD cultures during chondrogenesis.

Discussion

Role of DNA methylation during *in vitro* chondrogenesis

Regulatory pathways of DNA methylation play an important role in the degenerative diseases of articular cartilage. Recent studies point out DNA methylation as a potential therapeutic target for several human joint disorders, including OA. Stem cell-based therapies represent a very attractive component in the toolkit of regenerative medicine, thus a better understanding of DNA methylation during chondrogenesis is essential. To this end, we investigated the temporal expression pattern of specific regulators of DNA methylation at the mRNA level in different murine chondrogenic models, and studied the effects of the DNA methylation inhibitor 5-azaC on chondrocyte differentiation.

The gene expression pattern of three DNA methylation-associated genes (*Dnmt3a*, *Tet1*, *Ogt*) was examined in mouse cell line-based chondrifying micromass cultures. Of all the DNA methyltransferases, the importance of DNMT3B enzyme in normal limb development has already been proven. According to the results of PCR array, the expression of *Dnmt3a* and *Dnmt3b* genes showed strong elevation as chondrogenesis proceeded into later stages, especially during maturation and hypertrophy of chondrocytes. Recently published studies explained that TET enzymes might be key epigenetic regulators of chondrogenesis. Knockdown of *Tet1* induced significant downregulation of the cartilage matrix-specific genes *Col2a1* and *Acan* examined by *in vitro* experiments. In terms of the spatiotemporal distribution of TET enzymes in the developing vertebrae of mouse embryos, TET1 was the only protein that was detectable during the entire procedure of chondrogenesis. *Tet1* showed intense upregulation with the PCR array during the differentiation/maturing and hypertrophic stages of chondrogenesis. TET proteins (especially TET1) are strongly associated with OGT, which is capable of regulating the biological activity of TET enzymes. Accumulation of proteins that were post-translationally modified by OGT induced chondrogenic processes, rearrangement of ECM components and appearance of prehypertrophic chondrocytes in a cell line-based *in vitro* chondrogenic model. Based on these data, we expected the upregulation of OGT during the hypertrophic transformational phase of chondrogenesis. The transcript level of *Ogt* started to rise from day 10 of culturing according to the PCR array, but the most prominent upregulation was detected on culturing day 15. *Dnmt3a* and *Tet1* transcript levels were significantly enhanced on culturing day 5 based on the RT-qPCR results with the cell line-based HD cultures, but the expressional peak of all three examined genes was detected on day 10, and the level of

upregulation was lower on day 15. This means that the transcriptional activity of the genes was decreased by the late phase of hypertrophy. The gene expression pattern of the three selected genes was also studied during the *in vitro* chondrogenic differentiation of mouse embryonic limb bud-derived chondroprogenitor cells. In primary micromass cultures, a moderately high *Dnmt3a* expression was detected on culturing day 3 (at the time of the commitment of chondrogenic cell), and a gradual decrease along with the progress of chondrogenesis was seen when RT-qPCR results were analysed. *Ogt* showed a constant level of expression, but it was downregulated by culturing day 15. The expression of *Tet1* showed significantly elevated levels compared to the other two genes of interest, with the highest transcript levels identified during the phase of proliferation and commitment. It is worth to mention that the cell line-based and primary chondrifying micromass cultures may distribute different dynamics of cartilage formation which can be attributed to the differences in the migratory ability of the differentiating cells. We also aimed to detect the expression of the three examined genes in cryosections of 15-day-old whole mouse embryos by *in situ* hybridization. *Tet1* again had the strongest expression pattern among the three examined epigenetic regulators. The significant role of TET1 in osteochondrogenic differentiation was confirmed by experiments in which the specific knockout of *Tet1* impaired the skeletal development of the mutant mice: the examined animals showed growth irregularities. Specific knockdown of *Tet1* in C3H10T1/2 cells altered chondrogenic differentiation and caused a decrease in expression of chondrogenic markers such as *Col2a1*. We have also investigated the effects of the DNA methylation inhibitor 5-azaC on maturing chondroprogenitors and mature chondrocytes, and the changes of their differentiation abilities. 5-azaC treatment has been documented to affect the multipotency of human bone marrow stroma-derived mesenchymal stem cells differently: the cells retained their multipotent capacity after one pulse with 5-azaC, but additional pulses resulted in a restricted differentiation potential with increased tendency for chondrogenic commitment of the cells. However, in a different study, the expression of chondrogenic marker genes was reported to be negatively affected in chondrogenic cell cultures established from human bone marrow stroma-derived mesenchymal stem cells that were stimulated with 5-azaC. Controversies of the reported results draw attention to adjusting the proper parameters: in addition to concentration, we should also pay attention to the number, duration, and timing of the treatment. We demonstrated that 5-azaC had a differentiation stage-dependent effect during *in vitro* chondrogenesis in the primary murine micromass model. The mRNA expression levels of *Sox9*, *Col2a1* and *Acan* significantly decreased when 5-azaC was applied during the early stages of chondrogenesis; however, we could not detect any hypermethylation in the promoter regions of the genes examined. 5-azaC-

mediated blockage of DNA methylation at a later stage of chondrogenesis induced increased expression of *Sox9* and *Acan*. The observed upregulated gene expression could be traced back to hypomethylation in the corresponding promoters indicating that DNA methylation directly controls the transcriptional activity of key factors of chondrogenesis.

Role of circadian clockwork during *in vitro* chondrogenesis

Circadian clock has an important role in maintaining the normal homeostasis of cartilage tissue and controlling the differentiation mechanisms of chondrocytes. It has also been proven that clock disruption in mature chondrocytes may lead to osteoarthritis-like alterations. It is widely known that the circadian clockwork is retained in cells cultured *ex vivo.* We chose to use primary micromass cultures established from mesenchymal cells isolated from the developing limb buds of chicken embryos because immortalized cell lines often have reduced tissue-specific functions. During the 6-day long culturing procedure, chondroprogenitor cells in the HD cultures differentiate into chondroblasts that produce cartilage-specific ECM. Synthesis of cartilage ECM usually begins on culturing days 2 to 3. Our aim was to investigate the synchronized expression pattern of clock genes during early and late stages of *in vitro* chondrogenesis, thus we synchronized the molecular clock with serum shock on culturing days 1 and 6. According to our results acquired from HD cultures synchronized on day 1, the chondroprogenitor mesenchymal cells differentiating to early chondroblasts express all core circadian clock genes during early cartilage formation, and their expression followed a synchronized, rhythmic pattern. We discovered an approx. 8-hour phase angle difference between the positive regulator *BMAL1* and the negative regulators *CRY2-PER2-PER3* of the clock. In more mature and differentiated (6-day-old) serum-synchronized micromass cultures, we observed a transcriptional pattern similar to what we have seen in cultures synchronized on the first day of culturing. A sinusoid pattern with an approx. 8-hour phase angle between *BMAL1* and *PER2*/*CRY1* was detected, which means that the molecular clock functions properly at the mRNA level. These results indicate that in addition to immature mesenchymal cells, the more mature, ECM-producing chondroblasts and early chondrocytes are also characterized by a functional clock, which has already been described in mature chondrocytes cultured *in vitro*. The periodicity of the circadian rhythm dynamics in both early and late phase micromass cultures was similar to the previously published data of the circadian clock in mesenchymal stem cells isolated from adult tissues and mature chondrocytes. The key chondrogenic transcription factor (*SOX9)* and two major genes coding the components of

cartilage ECM proteins and proteoglycans (*COL2A1* and *ACAN*) also showed rhythmic expression patterns after synchronization with serum shock. The expression of circadian clock marker genes has been described in cartilage samples isolated from rodent laboratory animals, and the synchronized oscillatory expression pattern of *Sox9*, *Col2a1* and *Acan* caused by dark/light stimulus has also been detailed. Light/dark stimulation is not the only factor for clock synchronization, the effect of other factors such as the amount of nutrient is also important. We have proved that the gene products of the examined cartilage markers displayed a rhythmic expression pattern after synchronization with serum shock either on the first or the sixth day of culturing. In addition, micromass cultures serum-synchronized on the first day of culturing appeared to produce more abundant cartilage ECM by day 6. This chondrogenesis-promoting effect was also detectable at the transcription level of the marker genes *SOX9*, *ACAN*, and *COL2A1*. We concluded that synchronization with serum shock during the early phase of chondrogenesis affected *in vitro* cartilage formation in a positive way. This phenomenon was likely attributable to the synchronizing effect of serum shock on the molecular clockwork, because the co-application of the clock-modulator LDS suppressed the stimulating effects of serum shock on ECM production and chondrogenic marker gene expression. Disruption of the molecular clock in early chondrogenic cultures had a negative effect on cell proliferation, as we detected significantly lower cellular viability following treatment with LDS. We discovered a downward trend in transcript levels and the lack of a circadian oscillatory expression profile for *RUNX2*, the key transcription factor for osteogenic differentiation, after synchronizing the cells of the micromass cultures with serum shock on culturing day 1 or 6. The attenuated *RUNX2* transcript levels after serum shock may indicate a preferential differentiation of chondroprogenitors and chondrocytes toward the chondrogenic lineage. We managed to describe a serum shock-based experimental method for circadian clock synchronization, which facilitates the synchronization of the molecular clock components at cellular level, induces the chondrogenic commitment of differentiating chondroprogenitors, and enhances cartilage formation during *in vitro* chondrogenesis.

Summary

The overall aim of the research presented in this work was to identify the potential regulatory roles of DNA methylation and circadian clockwork during *in vitro* chondrogenesis. The most important results of this thesis are listed below:

Novel findings related to DNA methylation:

- The expression patterns of the three DNA methylation-associated genes *Dnmt3a*, *Tet1* and *Ogt* was altered depending on the developmental stage of cartilage formation.
- *Tet1* showed the most prominent expressional changes in all of the experimental models. *Tet1* may have a specific role during chondrogenic differentiation, and it may serve as a potential target protein for therapeutic agents affecting cartilage formation.
- 5-azacytidine treatment applied during the late stage of chondrogenesis caused an upregulation in the expression of chondrogenic marker genes *Sox9* and *Acan*. This was a direct result of inhibiting DNA methylation, because the promoter regions of the two chondrogenic marker genes showed hypomethylation. These promoter regions may indicate important regulatory regions for future epigenetic-associated treatments. 5-azacytidine may also be a feasible therapeutic agent in the near future.

Novel findings related to circadian clockwork:

- Serum shock-induced clock-synchronization caused a significant increase in the production of cartilage-specific extracellular matrix and upregulated the expression of chondrogenic marker genes *SOX9*, *ACAN* and *COL2A1*. Thus, we can conclude that serum shock promotes chondrogenic differentiation.
- Serum shock triggered a synchronized expression pattern of the core regulatory elements of the molecular circadian clock. We have shown the oscillating expression of the positive regulator *BMAL1* and the negative regulator *PER2/PER3/CRY1/CRY2*, indicating an antiphasic change at every 8 hours.
- Our results demonstrated that the alteration of circadian clock with longdaysin had a negative effect on cartilage formation, indicating that the proper rhythm of the circadian clockwork has a key positive role in the regulation of chondrogenesis.

List of publications

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Registry number: DEENK/212/2022.PL
Subject: PhD Publication List

Candidate: Judit Vágó
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10067066

List of publications related to the dissertation

1. **Vágó, J.**, Kiss, K., Karanyicz, E., Takács, R. Á., Matta, C., Ducza, L., Rauch, T. A., Zákány, R.: Analysis of Gene Expression Patterns of Epigenetic Enzymes Dnmt3a, Tet1 and Ogt in Murine Chondrogenic Models.
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List of other publications

3. Hajdú, T., Kovács, P., Zsigrai, E., Takács, R. Á., **Vágó, J.**, Cho, S., Sasi Szabó, L. A., Becsky, D., Keller-Pintér, A., Emri, G., Rácz, K., Reglődi, D., Zákány, R., Juhász, T.: Pituitary Adenylate Cyclase Activating Polypeptide Has Inhibitory Effects on Melanoma Cell Proliferation and Migration In Vitro.
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5. Matta, C., Juhász, T., Fodor, J., Hajdú, T., Katona, É., Somogyi, C., Takács, R. Á., **Vágó, J.**, Oláh, T., Bartók, Á., Varga, Z., Panyi, G., Csernoch, L., Zákány, R.: N-methyl-D-aspartate (NMDA) receptor expression and function is required for early chondrogenesis.
Cell Commun Signal. 17 (1), 1-19, 2019.
DOI: http://dx.doi.org/10.1186/s12964-019-0487-3
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