

Article

Modulation of DNA methylation influences cartilage formation in murine chondrogenic models

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Abstract: The aim of this study was to investigate the role of DNA methylation in the regulation of *in vitro* and *in vivo* cartilage formation. Based on the data of an RNA chip-assay performed on chondrifying BMP2-overexpressing C3H10T1/2 cells, the relative expression of *Tet1* (tet methylcytosine dioxygenase 1), *Dnmt3a* (DNA methyltransferase 3) and *Ogt* (O-linked N-acetylglucosamine transferase) genes was examined with RT-qPCR in mouse cell-line based and primary micromass cultures. RNA probes for *in situ* hybridization were used on frozen sections of 15-day-old mouse embryos. DNA methylation was inhibited with 5-azacytidine during culturing. We found very strong but gradually decreasing expression of *Tet1* throughout the entire course of *in vitro* cartilage differentiation along with strong signals in the cartilaginous embryonic skeleton. *Dnmt3a* and *Ogt* expressions did not show significant changes with RT-qPCR and gave weak *in situ* hybridization signals. Inhibition of DNA methylation applied during early stages of differentiation reduced cartilage-specific gene expression and cartilage formation. In contrast, it had stimulatory effect when added to differentiated chondrocytes. Our results indicate that the DNA demethylation-inducing *Tet1* is a significant epigenetic factor of chondrogenesis, and inhibition of DNA methylation exerts distinct effects in different phases of *in vitro* cartilage formation.

Keywords: Chondrogenesis; chondrocyte; cell differentiation; C3H10T1/2; high density culture; mouse embryo; epigenetic signals; DNA methylation; 5-azacytidine

1. Introduction

Epigenetics refers to those reversible and heritable biological processes that regulate gene expression without the alteration of the primary DNA sequence [1]. Beside genetic factors, environmental determinants also influence the development of an organism and the differentiation of cells. Epigenetic regulation is responsible for cell-specific gene expression and for the inheritance of these unique expression patterns to daughter cells [2]. The primary target of epigenetic processes is the DNA-histone complex termed chromatin. The accessibility of a specific DNA segment for transcription factors is influenced by different epigenetic marks such as DNA methylation or histone modifications. miRNA interactions are recently discovered epigenetic factors that may alter the transcriptional regulation of mRNA [3]. DNA methylation, the most widely studied epigenetic modulation, causes gene repression or silencing by adding a methyl group from the ubiquitous methyl donor S-adenosyl methionine to the carbon 5 position of cytosine rings of the DNA. The newly formed unit is called 5-methylcytosine (5-mC). This process is catalysed by DNA methyltransferases (DNMTs) which can be classified into two groups according to their enzymatic activity. *Dnmt3a* and *Dnmt3b* are *de novo* methyltransferases that have a role in generating new methylation patterns during ontogenesis. *Dnmt1*, however, has

the ability to transfer the already existing methylation motifs during cell division, thus it is referred to as a maintenance protein [4] [5]. Methylation sites are exceptionally frequent in the promoter regions of genes because they contain numerous CpG-sites. Transcription factors are unable to bind to their sites in case they are methylated [6]. Mature cells preserve their DNA methylation characteristics, while differentiating cells during ontogenesis can be modulated by demethylating factors in order to recover the pluripotent characteristics [7]. DNA demethylation is organized chiefly by proteins of the ten-eleven translocation methylcytosine dioxygenase (TET) family, which oxidise the methyl group of the 5-mC to 5-hydroxymethyl cytosine (5-hmC), hence reversing the effect of DNMTs and causing hypomethylation [8] [9]. Recent findings in murine embryonic stem cells confirmed that *Tet1* and *Tet2* proteins are strongly associated with the O-linked N-acetylglucosamine (O-GlcNAc) transferase (*Ogt*) and they act as a complex to maintain the unmethylated CpG-rich DNA regions [10].

Epigenetic regulation is essential during both physiological and pathological cartilage formation. DNA methylation and histone deacetylation are the most widely studied epigenetic mechanisms in relation to this developmental process [11] [12] [13] [14]. Early-stage chondrocyte differentiation is controlled by different transcription factors. As an example, SRY-box containing gene 9 (*Sox9*) is considered as the key transcription factor of chondrogenesis and it is necessary to regulate the expression of cartilage-specific extracellular matrix (ECM) genes [15]. The promoter regions of *Sox9*, as well as that of the RUNX family transcription factor 2 (*Runx2*), the master transcription factor of osteogenesis, exhibited a hypomethylated pattern in human synovium-derived mesenchymal stem cells (MSCs) during *in vitro* chondrogenesis [16]. The cartilage matrix-specific marker gene collagen type II alpha 1 chain (*Col2a1*) was also less methylated in chondrocytes in comparison with fibroblasts [17]. Conversely, collagen type X alpha 1 chain (*Col10a1*), a marker for hypertrophic chondrocytes, was confirmed to be methylated in human articular chondrocytes and its expression was initiated only after demethylation [18]. In a recent publication, *Tet1*-mediated *Sox9*-dependent activation of *Col2a1* and *Acan* during chondrogenesis has been demonstrated in ATDC5 cells [19].

Epigenetic modulations have also been observed in cartilage pathology. Osteoarthritis (OA) chondrocytes show characteristic methylation patterns, especially at the regulatory sites of genes encoding cartilage-specific transcription factors and ECM-components, matrix-degrading enzymes, and inflammation-related proteins [20]. In healthy hyaline cartilage, the expression of matrix metalloproteinases 3, 9 and 13 (*Mmp3*, *Mmp9* and *Mmp13*) is low; however, their promoter regions are less methylated in OA, which leads to their upregulation and subsequent ECM degeneration in damaged cartilage [21]. This type of demethylation is also characteristic for the aggrecan-degrading enzyme ADAM metalloproteinase with thrombospondin type 1 motif 4 (*Adams4*) [22]. Chondrocytes in OA exist in an inflammatory micro-environment which, amongst other factors, is triggered by demethylation at the promoter region of the interleukin 1 beta (*Il1β*) gene [23] [24]. The promoter of *Sox9* shows increased methylation which indicates the downregulation of *Sox9* in OA chondrocytes [25].

Methylating and demethylating proteins also play a crucial role in chondrocyte differentiation. DNMTs might serve as a promising epigenetic regulatory mechanism in cartilage repair [26]. *Dnmt3b* and *Tet1* were also recognized as significant epigenetic factors in chondrocyte differentiation, transcriptional control of cartilage-related genes and hypertrophic differentiation of chondrocytes [27] [28]. 5-azacytidine (5-azaC) is a chemical compound with the ability to modify epigenetic processes. It acts as a chemical analogue of the DNA nucleoside cytidine and has the ability to inhibit DNA methyltransferases [29]. 5-azaC is a potential antitumor and mutagenic agent that serves as a promising chemotherapeutical treatment option [30]. 5-azaC significantly promoted the osteogenic differentiation of adult bone marrow-derived murine MSCs [31], which indicates that it may be suitable for targeted control of stem cell differentiation into a desired cell type, for example chondrocytes.

Despite the accumulating wealth of data regarding the epigenetic regulation of gene activity in immature and mature cartilage, there are still many unanswered questions. The impact of epigenetic mechanisms on early stages of chondrogenesis and chondrocyte differentiation has not been described thoroughly, despite their high therapeutic relevance [32] [33] [34] [35]. In this study, we investigated the possible involvement of several epigenetic factors influencing DNA methylation in the regulation of chondrogenesis. We compared data obtained from chondrifying cultures of the murine embryonic mesenchymal cell line C3H10T1/2, murine primary chondrogenic cell cultures, and sections of developing whole mouse embryos. We performed a detailed expression analysis of *Dnmt3a*, *Tet1* and *Ogt* genes and investigated the impact of the targeted inhibition of DNA methylation on chondrogenesis by using 5-azaC. Our results indicated *Tet1* as a prominently expressed gene during both *in vitro* and *in vivo* chondrogenesis, and a developmental stage-dependent effect of 5-azaC, reflecting on the importance of DNA methylation and demethylation processes during cartilage formation.

2. Materials and Methods

EXPERIMENTAL MODELS

1. Primary chondrifying micromass cultures

High density cultures were established from mouse limb bud-derived mesenchymal cells following a protocol used on chicken micromass cultures with some modifications [36] [37]. First, NMRI laboratory mice were mated overnight. On the following day, successful mating was detected by confirming the presence of the vaginal plug – this day was considered as day 0 of gestation. Embryos on gestational day 11.5 (E11.5) were retrieved from the uterus. NMRI mice were sacrificed according to the ethical standards defined by the University of Debrecen Committee of Animal Research (Permission No. 2/2018/DE MÁB). After some brief washes with sterile calcium and magnesium-free phosphate buffered saline (CMF-PBS), distal parts of fore and hind limb buds were removed and pooled in sterile CMF-PBS. Limb buds were then dissociated in 0.25% trypsin-EDTA (Merck) incubated at 37 °C in a CO₂ incubator (5% CO₂, 80% humidity) for 20-30 min. After the addition of equal volume of foetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), cells were centrifuged for 10 min at 800 × g. The digested cells were filtered through a 40-µm pore size plastic filter unit (Corning, Tewksbury, MA, USA) in order to gain a single cell suspension of mesenchymal cells. Cells were centrifuged again for 10 min at 800 × g. The cell pellet was resuspended in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (*v/v*) FBS, 0.5 mM stable L-glutamine (Sigma-Aldrich), and antibiotics/antimicrobials (penicillin, 50 U/mL; streptomycin, 50 µg/mL; fungizone, 1.25 µg/mL; TEVA, Debrecen, Hungary) at a concentration of 1.0×10^7 cells/mL and 100-µL droplets were inoculated into 35 mm plastic tissue culture dishes (Eppendorf, Hamburg, Germany). Cells were allowed to attach to the surface for 2 h at 37 °C in a CO₂ incubator. Finally, 2 mL of culture medium was added into the culture dishes. Cultures were incubated in a conventional cell culture incubator. Day of inoculation was considered as day 0 of culturing. Cell cultures were maintained for 0, 1, 3, 4, 6, 10 or 15 days. The medium was changed on every second day.

2. Micromass cultures established from C3H10T1/2 BMP-2 cells

The BMP-2 overexpressing C3H10T1/2 cells were maintained as monolayer cultures in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% (*v/v*) FBS, 0.5 mM stable L-glutamine, 6.6 µg/mL ampicillin, 100 µg/mL streptomycin (TEVA), 5 µg/mL puromycin (Sigma-Aldrich), and kept in a humidified CO₂ incubator at 37 °C. Once monolayer cultures reached an approx. 80% confluence, cells were harvested in order to establish high density cultures, as described previously [38]. After trypsinisation, cells were centrifuged at 800 × g for 10 min. The number of cells was determined with the help of an automated cell counter (LUNATM, Logos Biosystems, Annandale, VA, USA). For micromass cultures, cell density was 1.0×10^7 cells/mL.

100- μ L droplets of the cell suspension were inoculated into 35 mm plastic Petri dishes (Eppendorf). This day was considered as day 0. After leaving the cells to attach to the surface (2 h, 37 °C), high glucose DMEM was added to the dishes, which was changed on every second day. The micromass cultures were maintained for up to 0, 5, 10, 15 days.

PCR ASSAY

Quantitative PCR arrays were used to monitor differential expression of 80 genes encoding epigenetic factors and chondrogenesis markers in C3H10T1/2 BMP-2 high density cell cultures. Total RNA was isolated using Direct-Zol® RNA Miniprep kit (Zymo Research, Irvine, CA, USA) from cultures harvested on designated days of culturing, and cDNA synthesis was conducted by iScript RT Supermix kit (Bio-Rad, Hercules, CA, USA) on 1 μ g total RNA. PrimerQuest software was used to design primers and were manufactured by Integrated DNA Technologies (Coralville, IA, USA). RT-qPCR was performed using a CFX RT-PCR machine (Bio-Rad) and the SsoFastEvaGreen™ Supermix (Bio-Rad). Parameters of PCR were set according to the manufacturer's protocol. The RT-qPCR primer sequences are in **Table S1** in the Supporting information. The specificity of the PCR primers was monitored by a post-PCR melting curve analysis, and heatmap analysis was conducted by CFX Maestro software (Bio-Rad).

RNA ISOLATION AND REVERSE TRANSCRIPTION

On the designated days of culturing, micromass cultures were washed with physiological NaCl two times and stored at -80 °C. Total RNA from C3H10T1/2 BMP-2 high density cell cultures and primary micromass cultures were isolated as previously described [39]. Briefly, cultures were mixed with TRI Reagent (Applied Biosystems, Foster City, CA, USA). 20% RNase-free chloroform was added and samples were centrifuged at 10,000 \times g at 4 °C for 15 min. After the incubation in 500 μ L RNase-free isopropanol for 1 h at -20 °C, total RNA was dissolved in RNase-free water and stored at -80 °C. RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription reactions were performed on 1000 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA was stored at -20 °C.

QUANTITATIVE REAL-TIME PCR ANALYSES

RT-qPCR reactions were performed as described previously [40]. The SYBR Green-based system (Promega, Madison, WI, USA) was used by absolute quantification using the standard curve method. Specific primer pairs for the chondrogenic and DNA methylation-specific genes were designed by the Primer-BLAST service of NCBI and ordered from Integrated DNA Technologies (IDT, Coralville, IA, USA). Nucleotide sequences of the primer pairs are shown in **Table S2** in the Supporting information. Conventional PCR was used to create standard curves for the absolute quantification. The Promega GoTaq Flexi DNA Polymerase kit (Promega, Madison, WI, USA) was applied to set up the following mixture (50 μ L per each reaction): 1.25 U GoTaq polymerase; 3 mM MgCl₂; 0.2 mM dNTP; 200 nM primers; and 50 ng cDNA (pooled from 6-day-old untreated samples). Amplification was performed in a programmable thermal cycler (MultiGene 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) using the following thermal profile: initial denaturation (95 °C, 5 min), followed by 40 cycles of denaturation (95 °C, 15 s), annealing (58 °C, 20 s), extension (74 °C, 20 s); and final extension (74 °C, 5 min). Roche High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) was used to purify the PCR products according to the instructions of the manufacturer. DNA concentration of the isolated PCR products was detected using a Nanodrop 1000 UV-Vis spectrophotometer (Thermo Fisher Scientific). Purified samples were diluted in a serial manner (10-fold, starting with 1 ng/ μ L) to establish the standard curves. The QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) was applied for the reactions using the GoTaq qPCR Master Mix (Promega) and 10 ng cDNA per each 10- μ L reaction. The settings for the process were the following: initial denaturation (95 °C, 2 min), followed by 40 cycles

of denaturation (95 °C, 5 s), annealing and extension (60 °C, 30 s), and final extension (72 °C, 20 s). Amplification was followed by a melt curve stage consisting of 3 steps: denaturation at 95 °C for 15 sec, annealing at 55 °C for 15 sec, and a dissociation step at 0.15 °C/sec increments between 55 °C and 95 °C. Amplification data were analysed using the QuantStudio Design and Analysis Software (version 1.5.1) and exported data were processed using Microsoft Excel.

DIGOXIGENIN-LABELLED RNA PROBE PREPARATION

PCR primers were designed to amplify a ~1000 bps long region from the 3'UTR of *Dnmt3a*, *Ogt* and *Tet1* genes. PCR-amplified 3'UTR regions were cloned into pDrive vector (Qiagen, Germantown, MD, USA) and sequenced. Insert flanking T7 promoters were used for generating antisense probes. Sequence data of the cloned regions are in **Table S3** in the Supporting information. The specific gene products of *Dnmt3a*, *Ogt* and *Tet1* probes were amplified with the help of PCR from the plasmids. Amplifications were performed in a thermal cycler (Labnet MultiGene™ 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) using the following settings: 95 °C, 2 min, followed by 33 cycles (denaturation, 95 °C, 15 s; annealing for 20 s at 57 °C; extension, 72 °C, 75 s) and then 72 °C, 2 min. Digoxigenin-labelled RNA probe preparation was performed as recommended by Roche, with some modifications. The amplified PCR products were isolated using a Roche High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) according to the instructions of the manufacturer. DNA concentration of purified PCR products were detected with the help of a Nanodrop 1000 UV-Vis spectrophotometer (Thermo Fisher Scientific). The specific RNA labelling was developed with a DIG RNA labelling mix by *in vitro* transcription of DNA. First, the following components were mixed together to create the DIG RNA labelling mix: 1 µL of purified PCR product (concentration between 100-200 ng/µL); 2 µL of 10× concentrated DIG RNA Labelling Mix (Promega); 4 µL 5× Transcription Buffer (Promega); 2 µL 100 mM Dithiothreitol (DTT) (Promega); 2 µL T7 RNA Polymerase (Promega) and 9 µL nuclease-free water (NFW) (Promega) to make a total reaction volume of 20 µL. After the components were mixed together, the mixture was incubated for 2 h at 37 °C. Polymerase reaction was terminated by 2 µL 0.2 M EDTA (pH 8.0). The labelled RNA was precipitated after the addition of 2.5 µL 4 M LiCl and 75 µL pre-chilled 100% ethanol. After a brief mix with vortex, the precipitate was incubated at -80 °C overnight. On the next day, the sample was centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was discarded, and the pellet was washed with 100 µL ice-cold 70% (*v/v*) ethanol. The precipitate was centrifuged again at 13,000 × g for 15 min at 4 °C, and after discarding the supernatant, the sample was left to dry on room temperature for some minutes. Finally, the RNA pellet was dissolved in 75 µL hybridization buffer (containing 20× saline sodium citrate (SSC), dextran sulfate, 50× Denhardt's solution, sodium dodecyl sulfate (SDS), tRNA, and 50% (*v/v*) formamide; Sigma-Aldrich) and stored at -20 °C.

IN SITU HYBRIDIZATION

Whole murine embryos were collected as previously described. Briefly, NMRI mice were mated overnight, and detectable vaginal plug confirmed on the following morning, which was regarded as day 0. On gestational day 15, whole mouse embryos were retrieved from the uterus, washed in DEPC-PBS (PBS with 0.1% diethylpyrocarbonate) and were fixed in 4% paraformaldehyde (PFA, dissolved in DEPC-PBS) overnight. On the following day, embryos were washed in DEPC-PBS two times for 10 min each, then immersed into 15% and 30% RNase-free sucrose solution until they sank. After embedding the embryos into Cryomount medium (Bio-Optica, Milan, Italy), 20 µm thick frozen sections were cut in a sagittal plane using a cryostat (CM3050 S, Leica Biosystems, Buffalo Grove, IL, USA) and mounted onto Superfrost glass slides (Thermo Fisher Scientific). Sections were stored at -20 °C. We applied a nonradioactive *in situ* hybridization protocol described earlier, with some modifications [41]. Briefly, sections were removed from -20 °C and left at room temperature for 20 min. The glass slides were placed into a 58 °C

incubator overnight for drying. On the following day, slides were removed from the incubator and left at room temperature for 20 min. Samples were fixed in 4% PFA (dissolved in DEPC-PBS) for 20 min. After washing with DEPC-PBS for 2×10 min, the remaining liquid was blotted and samples were treated with 100 µL Proteinase K solution (20 µg/mL; Promega) at 37 °C for 20 min. The slides were washed with DEPC-PBS for 2×5 min. Samples were prehybridised for 4 h at 58 °C, then the solution was changed to the hybridisation solution that contained the RNA probe (1–2 µg/mL) and the slides were incubated at 58 °C for 16 h. All components were RNase free until this step. On the third day, slides were washed in 1× SSC at 58 °C for 15 min, then in 1.5× SSC for another 15 min at 58 °C, and finally twice in 2× SSC for 2×20 min at 37 °C. Samples were treated with 0.5 µg/mL RNase A dissolved in 2× SSC at 37 °C for 20 min. After washing in 2×SSC at room temperature for 10 min, slides were washed twice in 0.2× SSC at 58 °C for 2×30 min. Then, sections were washed twice at 58 °C for 2×15 min, then at room temperature for 10 min with PBST. Finally, samples were incubated in 10% Blocking buffer solution (Blocking buffer powder dissolved in maleic acid buffer with Tween (MABT); Roche) with α -DIG antibody (anti-digoxigenin, 1:1000; Abcam, Cambridge, UK; Cat. No.: ab420) at 4 °C overnight. Sections were then washed three times in PBT (PBS with 0.1% Triton X-100 and 2 mg/mL BSA) for 3×20 min, then twice in 1 M TRIS solution (pH 9.0) for 2×5 min. Digoxigenin antibody was visualised by incubation with TRIS-NBT/BCIP solution (20 mg/mL stock solution of nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate, dissolved in 1 M TRIS; Sigma-Aldrich) at room temperature in the dark for 2–20 hours (depending on the amount of the RNA). After the incubation time, samples were washed in PBST for 2×10 min. Finally, slides were mounted with DPX medium (Sigma-Aldrich). Photomicrographs of the sections were taken using an Olympus BX53 camera on a Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan).

DIMETHYL-METHYLENE BLUE STAINING METHOD

The dimethyl-methylene blue (DMMB) staining method was used to demonstrate the amount of metachromatic cartilage ECM in mouse whole embryos and also in primary murine high density cultures. Sections of whole embryos stained with DMMB served as a control for *in situ* hybridization. Frozen sections were prepared as described above. After the glass slides were removed from –20 °C they were dried at room temperature for 10 min, then at 58 °C for 1 h. After washing in distilled water for 2×10 min, samples were stained with 0.1% (*w/v*) DMMB (Sigma-Aldrich) dissolved in distilled water for 5 min. Surplus dye was removed by washing the sections with distilled water for 3×10 min. Slides were mounted with DPX. Photomicrographs of the stained samples were taken as described above. As for HD cultures, 30-µL droplets of the cell suspensions were inoculated on the surface of 10-mm round coverglasses (Menzel-Gläser, Menzel GmbH, Braunschweig, Germany) into 24-well culture plates. On day 4 or 6 of culturing, colonies were rinsed with PBS and fixed in a 4:1 mixture of absolute ethanol and 40% formaldehyde. After rehydration in a descending series of ethanol, cultures were stained with 0.1% (*w/v*) DMMB dissolved in 3% (*v/v*) acetic acid (pH 1.8). Surplus dye was washed in acetic acid, then with distilled water. Finally, cultures were mounted with Aquatex (Sigma-Aldrich). Photomicrographs of the stained cultures were taken as described above.

MANIPULATION OF EPIGENETIC REGULATION WITH 5-AZACYTIDINE

The epigenetic modifier 5-azacytidine (5-azaC; Cat. No.: A2385; Sigma-Aldrich) was used to inhibit DNA methyltransferases and to consequently activate specific gene regions by triggering DNA demethylation [30] [42]. 5-azaC was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and then applied at a final concentration of 10 µM for 72 h on culturing day 1 or 3. Primary micromass cultures were harvested on the 4th or 6th day of culturing, according to the treatment protocol. Control colonies were treated with equal amount of the vehicle (DMSO).

MITOCHONDRIAL ACTIVITY (MTT) ASSAY

Cell viability was monitored as previously described [36]. 24-well plates were used for cell culturing. 25 μ L of MTT reagent (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide; 5 mg/mL in PBS) were pipetted into each well on culturing day 4 or 6. Cells were incubated for 2 hours at 37 °C. Following the addition of 500 μ L MTT solubilizing solution (10% Triton X-100 in 2-propanol), optical density was measured at 570 nm (Chameleon, Hidex Ltd., Turku, Finland). Measurements were carried out in 3 samples of each experimental group in 3 independent experiments. Optical density readings of the experimental groups have been normalized to those of the vehicle controls and shown as percentage changes.

CELL PROLIFERATION ASSAY WITH ^3H -THYMIDINE LABELLING

Rate of cell proliferation was examined as previously described [36]. Briefly, 1 μ Ci/mL ^3H -thymidine (diluted from methyl- ^3H -thymidine; 185 GBq/mM, Amersham Biosciences, Budapest, Hungary) was added to cell culture medium on day 3 or 5, 16 h before the end of treatments. After washing with PBS, proteins were precipitated with ice-cold 5% trichloroacetic acid, and washed with PBS again. Colonies were then air-dried for one week and radioactivity was counted by a liquid scintillation counter (Chameleon, Hidex). Measurements were carried out in 9 samples of each experimental group in 3 independent experiments. Scintillation counting data of the experimental groups were normalised to those of the respective controls and presented as percentage changes.

STATISTICAL ANALYSIS

All data are representative of at least three independent experiments. Data in figures is representative of the mean \pm SEM (standard error of the mean) of a single experiment. With regard to RT-qPCR reactions, one representative data set is shown out of 3 parallel experiments showing similar trends, and data were normalised to beta actin (*Actb*) or Succinate Dehydrogenase Complex Flavoprotein Subunit A (*SdhA*), as calculated by NormFinder. Statistical differences were determined using paired Student's *t*-test or One-Way ANOVA with Tukey HSD. The specific differences were considered statistically significant if $P < 0.05$. Statistical significance is indicated by asterisks as follows: $P < 0.05 = *$; $P < 0.01 = **$; $P < 0.001 = ***$.

3. Results

3.1. *Dnmt3a*, *Tet1* and *Ogt* epigenetic marker genes display a specific expression pattern in murine chondrogenic models

We first studied the expression pattern of epigenetic marker genes in different *in vitro* murine chondrogenic model systems. Samples for RNA assays were obtained from high density cultures established from C3H10T1/2 BMP-2 cells collected on culturing days 0, 5, 10 and 15 (corresponding to the main stages of chondrogenesis *in vitro*), in order to examine the expressional peaks of epigenetic markers at the mRNA level. The results of the RNA assay clearly showed the expression of every gene of interest (**Figure 1**). Interestingly, many of the epigenetic marker genes in connection with DNA methylation were upregulated at later stages of chondrogenic differentiation (culturing days 10 and 15). Three epigenetic modifiers were chosen for subsequent analysis: DNA methyltransferase 3 alpha (*Dnmt3a*), Tet methylcytosine dioxygenase 1 (*Tet1*), and O-Linked N-Acetylglucosamine (GlcNAc) Transferase (*Ogt*). *Dnmt3a* was strongly expressed on culturing day 15, while *Tet1* expression peaked around day 10. It is worth noting that *Ogt*, which interacts with *Tet1*, peaked on culturing days 10 and 15. On the other hand, the expression profile of the chondrogenic markers collagen type II alpha 1 chain (*Col2a1*) and aggrecan (*Acan*) showed an earlier activation and increase in transcript levels between days 5 and 10 of culturing. *Col10a1*, a marker for matrix mineralization and chondrocyte hypertrophy, showed an expressional peak in the samples on days 10 and 15. The expression pattern of *Col1a1* gene followed that of *Col10a1*, reflecting on the initiation of osteogenic differentiation in the presence of hypertrophic chondrocytes.

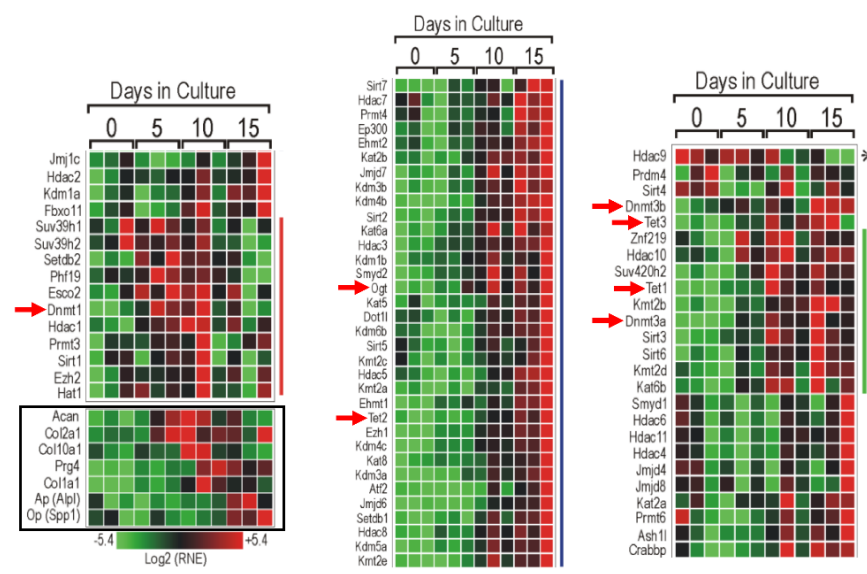


Figure 1. RNA assay with C3H10T1/2-BMP2 cells in chondrifying micromass cultures collected on designated days of *in vitro* cartilage formation. Chondrogenic differentiation-associated changes in the expression of epigenetic factors visualized with a heatmap. The red squares refer to the upregulated genes and the green squares indicate the downregulated genes. Genes next to the red line are mostly upregulated between the 5th and 10th days of culturing. Genes neighbouring the blue line are upregulated around culturing day 15. Genes next to the green line are upregulated between the 10th and 15th days of culturing. Specific DNA methylation and demethylation regulator genes are marked with red arrows. Data indicated with the black rectangle: expressional changes of chondrogenic and osteogenic marker genes in order to verify the cartilaginous differentiation of micromass cultures.

After RNA isolation from the cell-line based micromass cultures, quantitative real-time PCR analysis was carried out to study the relative expression of the three DNA methylation-specific marker genes during chondrogenesis. The mean quantity values for *Dnmt3a*, *Tet1* and *Ogt* markers were normalized to *Actb*. The largest increase of gene expression was on culturing days 10 and 15 for all three genes (**Figure 2**). The relative gene expression of *Tet1* displayed the most prominent changes, with the greatest degree of up-regulation on day 10 of culturing. The expression profiles showed high similarity to those detected with the RNA assay.

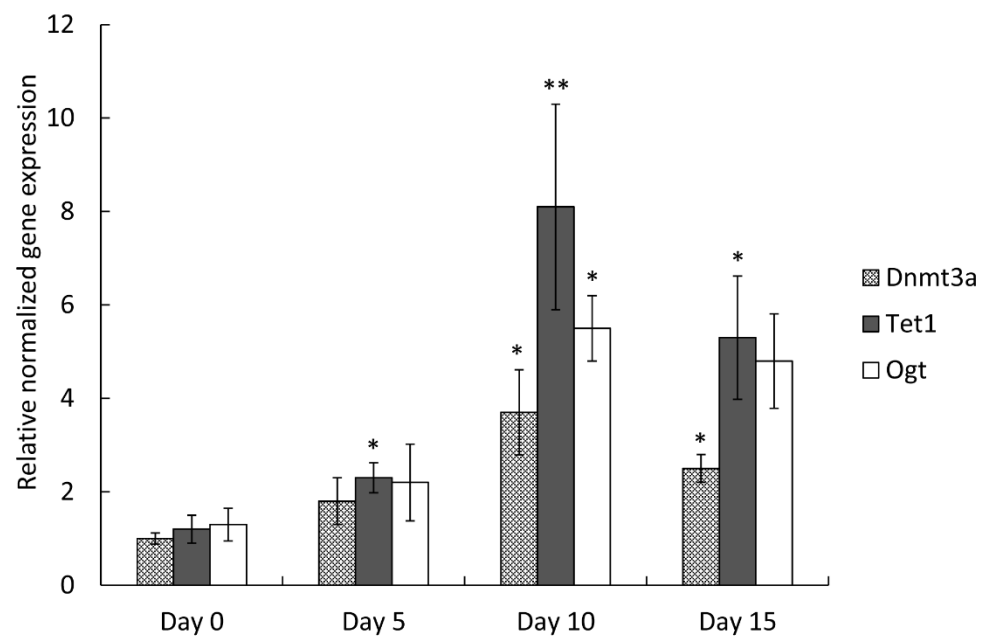


Figure 2. RT-qPCR analysis of *Dnmt3a*, *Tet1* and *Ogt* gene expression in differentiating cultures of C3H10T1/2 BMP2 cells, collected on culturing days 0, 5, 10 and 15. Measured C_T values were normalized to that of *Actb*. Mean \pm SEM and levels of significance (* $P < 0.05$, ** $P < 0.01$) are indicated. One-Way ANOVA with Tukey HSD was employed for evaluating significance. Representative results out of 3 independent experiments (biological replicates) showing similar trends of changes.

Primary high density cultures were established from mouse embryonic limb buds and collected on designated culture days. Transcripts for the DNA methylation genes were also identified in this type of *in vitro* model by RT-qPCR; however, their expression profile was more varied compared to the cell line-based model. After choosing the most stably expressed normalizing gene, the mean quantity values for the three examined DNA methylation markers were normalized to the reference gene *Sdha*, and the normalized mean quantity was set to 1.00 on culturing day 0 for each of the genes. *Tet1* showed the highest expressional fold change among the three examined genes, with peaks on day 1 and 3 of culturing. *Dnmt3a* transcript level was the highest on day 3 and displayed a significant downregulation by day 15. *Ogt*, on the contrary, was constantly expressed by the differentiating chondrocytes, except on day 15, when it was significantly downregulated (Figure 3).

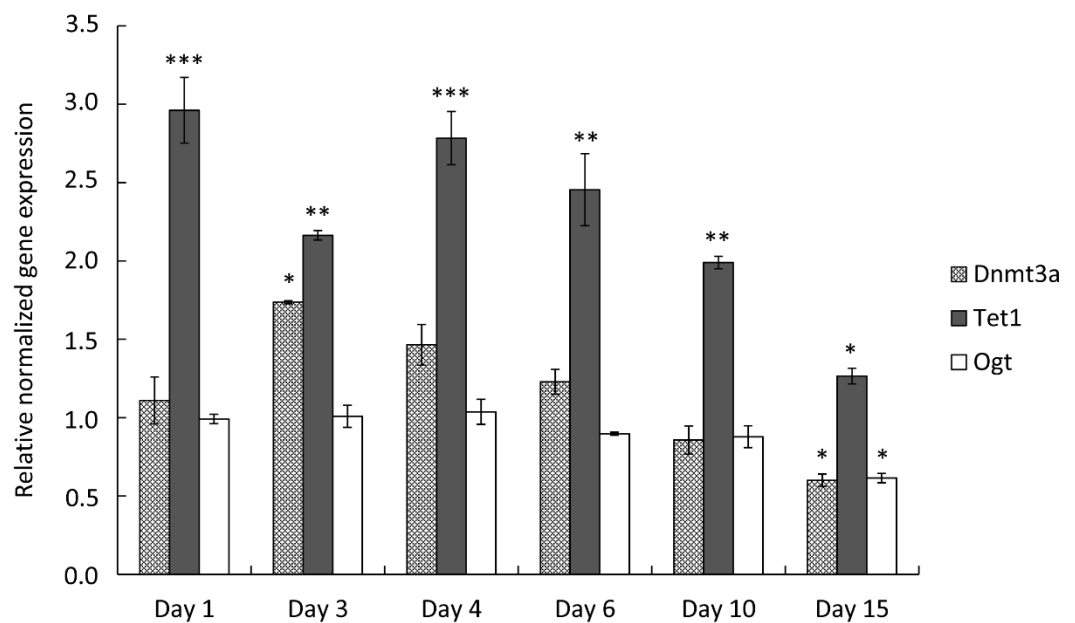


Figure 3. DNA methylation-specific marker gene expression in primary murine micromass cultures on various days of culturing during *in vitro* cartilage formation as determined by RT-qPCR. Data are expressed as the mean \pm SD relative to culturing day 0 and normalized against the reference gene *Sdha*. Statistically significant differences of gene expression levels are indicated by asterisks as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Representative results out of 3 independent experiments (biological replicates) showing similar trends of changes.

Next, we aimed to prove the *in vivo* relevance of the three examined genes in chondrogenesis *in vivo*. Therefore, we performed *in situ* hybridization analyses of *Dnmt3a*, *Tet1* and *Ogt* in 15-day-old whole mouse embryo samples. This experiment revealed that all three genes were expressed in the territories of hyaline cartilage islands of the developing forelimb, vertebrae and sternum of the E15 mouse embryos. Among the three examined genes, *Tet1* signals were the strongest (Figure 4. a, b, c), *Dnmt3a* showed a moderate expression pattern (Figure 4. d, e, f), while *Ogt* appeared to have a weak expression in the primitive limb and vertebrae compared to the other two epigenetic markers (Figure 4. g, h, i). At this developmental stage, cartilage had not started to differentiate into bone, the hypertrophic cartilaginous area was minimal, and ossification has not commenced yet but the cartilaginous primordia of the future bones were well visible. The purple metachromatic colour was obtained with the application of DMMB, in order to demonstrate the cartilage elements of the developing limbs, vertebrae, skull and ribs (Figure 4. j, k, l).

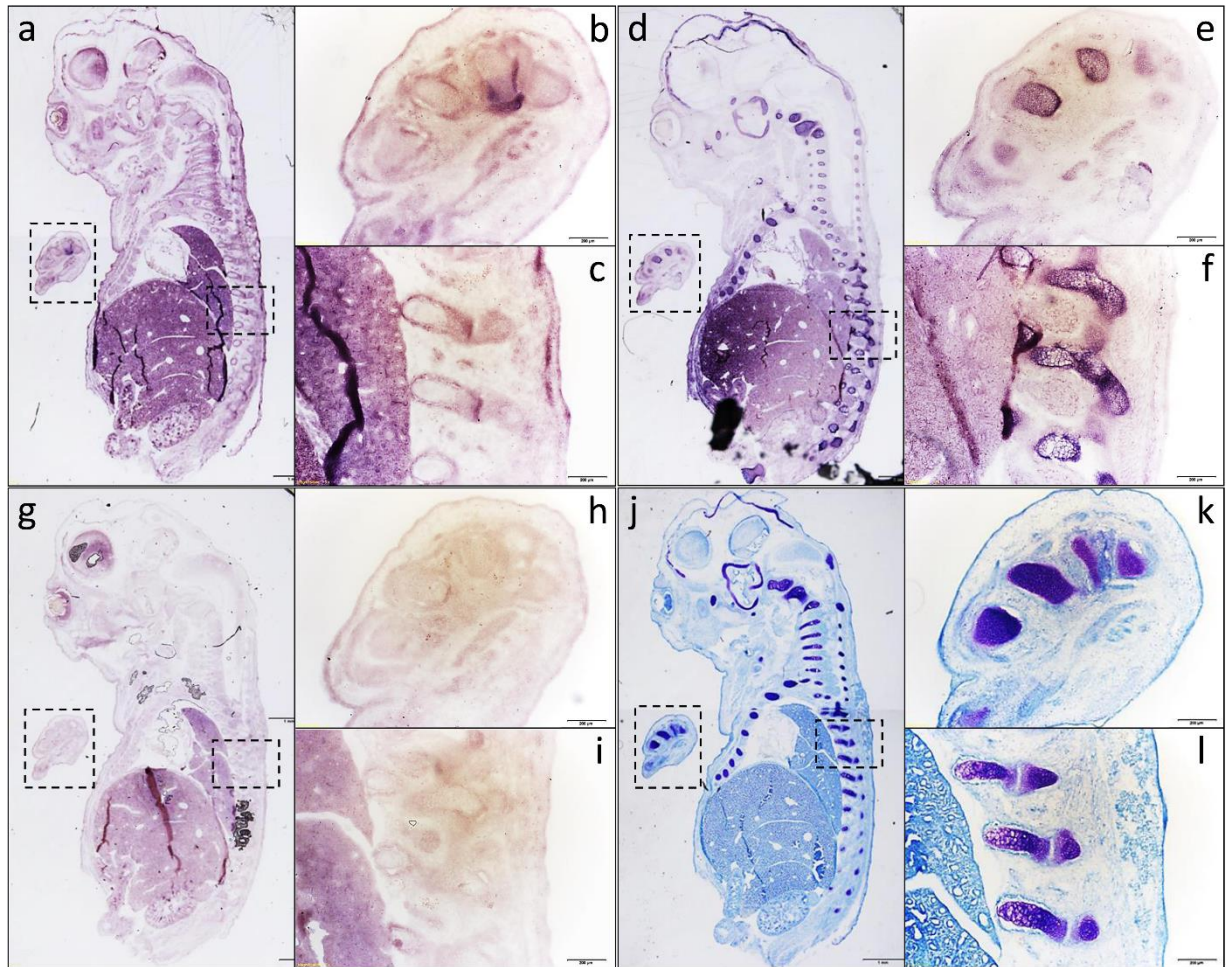


Figure 4. *In situ* hybridization analysis of epigenetic marker gene expression in E15 mouse whole embryos. Sagittal sections of frozen embryos were processed for *in situ* hybridization with RNA probes encoding *Dnmt3a* (a, b, c), *Tet1* (d, e, f) and *Ogt* (g, h, i). Sections were also stained with DMMB for cartilage-specific proteoglycans (j, k, l). Metachromatic (purple) areas in photomicrographs show polyanionic glycosaminoglycan-rich cartilage ECM. Photomicrographs of whole embryos were taken with a 4× objective (a, d, g, j). Inserts were taken with a 10× objective, which correspond to areas indicated with boxes (b, c, e, f, h, i, k, j). Note the strong expression of *Dnmt3a* and *Tet1* in maturing chondrocytes of the developing vertebrae, limb buds and skull in the mouse embryo. Scale bar for a-d: 1 mm, for the rest: 200 μm.

3.2. ECM morphology, cell proliferation and cell viability of early and late chondrogenic stages are different after 5-azaC treatment

The DNA methylation inhibitor 5-azaC was applied on primary murine micromass cultures at 10 μM. Some high density cultures were treated during the beginning of chondrogenesis (i.e. from day 1 for 72 hours), while other cultures were treated from day 3 for 72 hours in order to investigate its effects on later stages of chondrogenesis. To visualise cartilage-specific ECM accumulation in the primary murine micromass cultures, the qualitative DMMB staining method was used on culturing days 4 and 6 at the end of the treatment protocols. The DNA methylation inhibitor attenuated the amount of metachromatic ECM produced if applied at the early stage of chondrogenesis. Interestingly, when 5-azaC was administered from culturing day 3 for 72 hours, the morphology of metachromatic cartilage nodules was similar to that of the untreated HD cultures. It is of note that the characteristic metachromatic (purple) colour was weaker in the treated colonies at day 6, indicating that the chondrocytes of these cultures probably produced less ECM compared to the controls (**Figure 5. a**).

We hypothesized that one of the reasons behind the attenuated ECM production could be the altered proliferative and mitochondrial activity of the chondroprogenitor cells and chondrocytes. Thus, we examined the effects of 5-azaC on cell viability and cell proliferation during chondrogenic differentiation. The assays were carried out on culturing days 4 or 6, depending on the starting day of treatment. Both treatment methods inhibited the proliferation of chondrifying cells, especially during the early stages of chondrogenesis, when this parameter was lowered by 55% ($\pm 5\%$), as opposed to later stages, when the rate of cell division was reduced by 37% ($\pm 7\%$) (**Figure 5. b**). We also studied the potential cytotoxic effect of 5-azaC during *in vitro* cartilage formation. The percentage of viable cells in the 4-day-old colonies after treatment was 90% ($\pm 2\%$), compared to the control group, and this was a significant decrease. In contrast, cells in 6-day-old micromass cultures showed a massive reduction in their mitochondrial activity ($24 \pm 3\%$) (**Figure 5. c**).

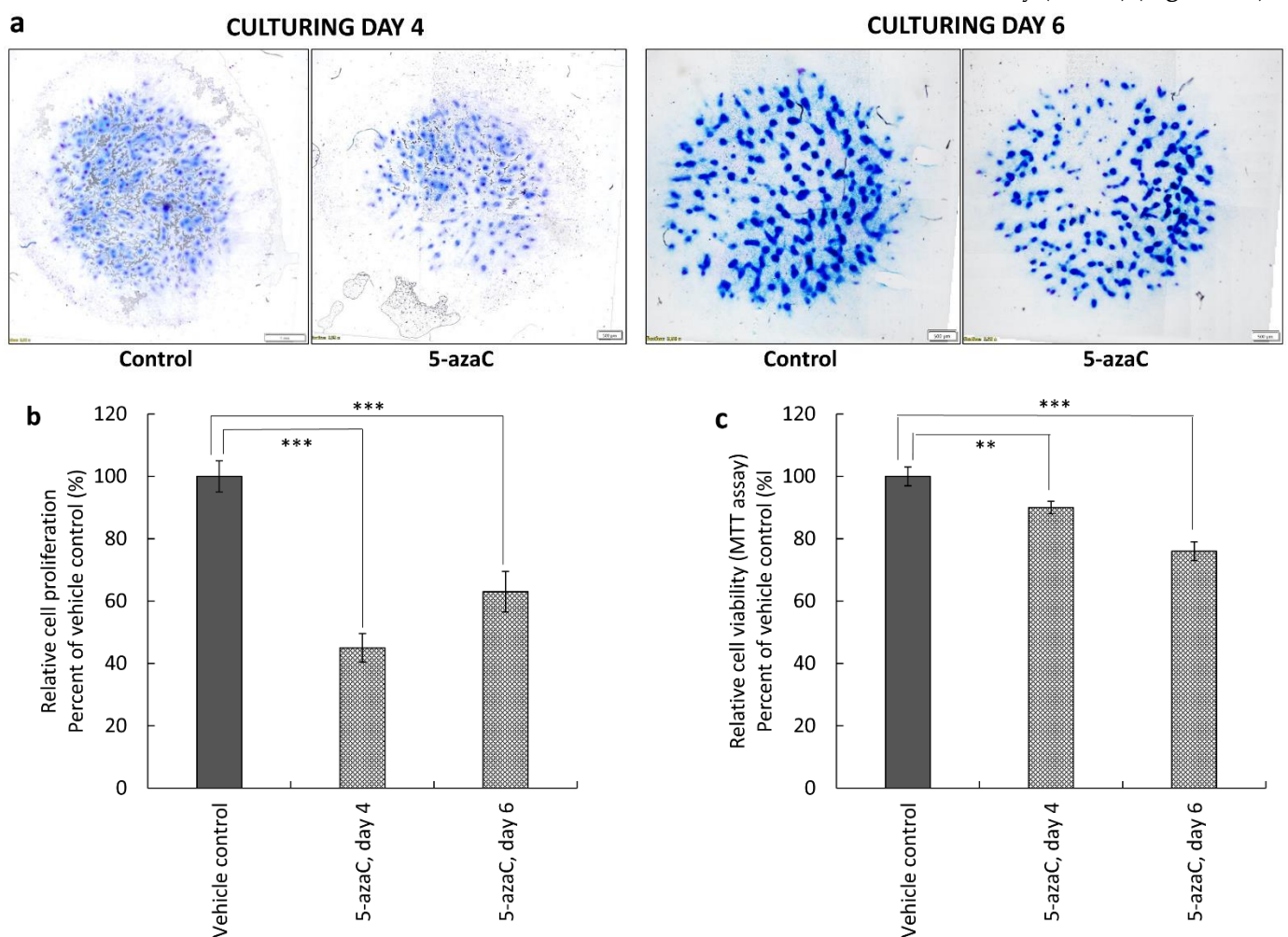


Figure 5. Effect of the DNA methylation inhibitor 5-azaC on cartilage ECM production, cell proliferation and cell viability. (a) Metachromatic staining of 4- and 6-day-old primary murine high density cultures. 5-azaC was applied from the first or the third day of culturing, respectively, for 72 hours at a final concentration of 10 μM . Metachromatic ECM accumulation was visualized by dimethyl methylene blue (DMMB) qualitative staining assay. Original magnification was 4 \times . Scale bar: 1000 or 500 μm . Effects of 5-azaC on cell proliferation (b) and cell viability (mitochondrial activity) (c) in primary murine high density cultures. Cell viability was determined by using MTT assay and cell proliferation was examined by ^3H -thymidine incorporation assay on day 4 or day 6, following treatment with 5-azaC. Statistically significant differences between proliferation rate and mitochondrial activity of cells in cultures that received the inhibitor *versus* vehicle control cultures are marked by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Representative data out of 3 independent experiments.

3.3. Inhibition of DNA methylation influences chondrogenic marker gene expression depending on the developmental stage of chondrogenesis

In order to detect the effects of 5-azaC treatment on gene expression profiles in primary micromass cultures, RT-qPCR reactions were performed. We collected samples for total RNA isolation on culturing days 4 or 6. 5-azaC was applied for 72 hours prior to the sample collection. First, we wanted to check whether the expression of the investigated genes mediating DNA methylation was altered after the application of the inhibitor. To this end, we assessed the quantitative expression profile of *Dnmt3a*, *Tet1* and *Ogt*. Our results confirmed that 5-azaC treatment significantly downregulated the expression of *Dnmt3a* and *Ogt*, while *Tet1* expression was not influenced. This pattern was similar in the two different experimental groups and reflected on a transcriptional influence of 5-azaC on *Dnmt3a* and *Ogt* genes (Figure 6. a).

Next, we studied the mRNA levels of key chondrogenic marker genes with RT-qPCR. The key chondrogenic transcription factor *Sox9*, as well as the two major cartilage matrix-specific genes (*Col2a1* and *Acan*) were selected. We found that the expression profiles of these genes were significantly altered after the inhibition of DNA methylation at both the early and the late stages of chondrogenesis (Figure 6. b). During the early stage of *in vitro* cartilage formation, all three marker genes were significantly downregulated. The largest decrease was detected for *Col2a1* and *Acan*. On the contrary, during the later stage of chondrogenesis, *Sox9* and *Acan* were upregulated, while *Col2a1* expression remained unchanged.

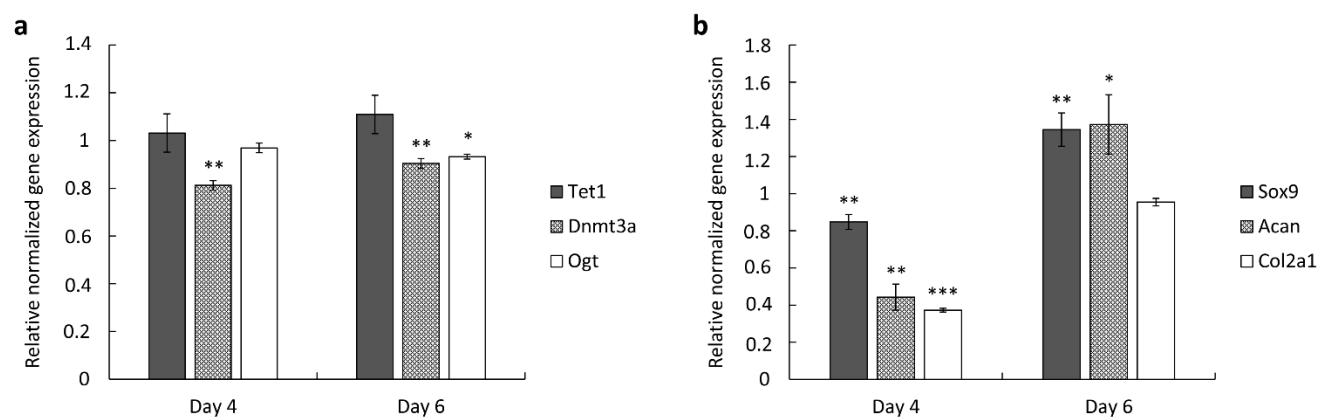


Figure 6. DNA methylation-specific (a) and cartilage-specific (b) marker gene expression in 4- and 6-day-old primary murine chondrifying cell cultures after 5-azaC treatment. The DNA methylation inhibitor was added to the culture medium from the first or the third day of culturing, respectively, for 72 hours at a final concentration of 10 μ M. Data are expressed as the mean \pm SD relative to the vehicle control and normalized against the reference gene *Sdha*. Statistically significant differences of gene expression levels are indicated by asterisks as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Representative data out of 3 independent experiments.

4. Discussion

DNA methylation plays an important role in joint diseases [43]. Recent studies indicate that DNA methylation may serve as a promising therapeutic target for several human joint disorders, including osteoarthritis [44]. Since the stem cell therapy-based approach represents a very attractive component in the toolkit of regenerative medicine, a better understanding of DNA methylation during early chondrogenesis is essential. To this end, we investigated the role of DNA methylation during *in vitro* chondrogenesis in different experimental models in the present study. We focused on the temporal expression pattern of specific regulators of DNA methylation at the mRNA level in different murine chondrogenic models, and we have also investigated the effects of the DNA methylation inhibitor 5-azaC on chondrocyte differentiation.

First, we studied the osteo-chondrogenic differentiation in high density cultures of the mesenchymal cell line C3H10T1/2 in which cells constitutively express BMP-2 [45]. The cell line-based high density cultures were collected for RNA isolation on designated days of culturing, based on the specific differentiation stage of chondrocytes. In terms of chondrogenic marker expression patterns, the results of the RNA assay are in a good correlation with our earlier study which analysed the transcript levels of the same markers by conventional RT-PCR [38]. The proteins coded by the *Col2a1* and *Acan* genes are characteristic components of the cartilage-specific ECM [46]. According to the RNA assay, these genes were upregulated around the 5th day of culturing, corroborating our earlier results suggesting that metachromatic areas corresponding to glycosaminoglycan-rich cartilage ECM started to appear from the 3rd day of culturing [38].

After having verified the expression of chondrogenic marker genes by the RNA assay in murine cell-line-based micromass cultures, we undertook to study the gene expression profiles of epigenetic markers. The importance of *Dnmt3b* enzyme in normal limb development and hypertrophic chondrocyte maturation has already been proven [14]. This was well visible with the RNA assay, where the expression of *Dnmt3a* and *Dnmt3b* genes showed strong elevation as chondrogenesis proceeded into later stages. TET enzymes contributing to the reversible nature of DNA methylation were also investigated, as recent studies pointed out that *Tet1* might be a key epigenetic regulator of chondrogenesis. Although lineage-specific knockdown of *Tet1* caused only minor skeletal abnormalities in the transgenic animals, significant downregulation of the cartilage matrix-specific gene expression was observed by *in vitro* experiments [19] [27] [47]. In line with these observations, *Tet1*, 2, and 3 showed intense expression in the heatmap of the RNA assay during the second half of *in vitro* chondrogenesis.

In addition to the cell-based model, we also employed a primary high density cell culture system established from murine limb bud-derived chondroprogenitor mesenchymal cells to study the formation of hyaline cartilage *in vitro* [48]. In primary micromass cultures, a moderately high *Dnmt3a* expression was detected at the time of the commitment of chondrogenic cells (i.e. day 3 of culturing) and a gradual decrease along with the progress of chondrogenesis was seen when RT-qPCR results were analysed. The expression of *Tet1* showed significantly elevated levels compared to the other two epigenetic genes of interest. *Ogt*, encoding a molecular partner of TET enzymes, showed low and constant level of expression as revealed with RT-qPCR. *Ogt* clearly has a specific association with the TET enzymes, as it has the same expression pattern as the three TET genes according to the RNA assay. The reason behind the different quantitative gene expression profiles between the cell line-based and primary micromass cultures could be the differentiation rate and state of the cells in the high density cultures. The C3H10T1/2 BMP2 cell-based micromass cultures demonstrated a distinct macroscopic morphology compared to the primary high density cultures on culturing day 6 according to our earlier results [38], which means that the chondrogenic differentiation of cell-line based cultures might be slightly slower. Additionally, primary micromass cultures represent a more heterogeneous cell population, in which low number of fibroblasts can also be present.

Next, we aimed to detect the expression of the three examined epigenetic markers at the mRNA level in cryosections of 15-day-old whole mouse embryos by *in situ* hybridization. We have chosen the age of the embryo by considering the stages of cartilage development as described by Rafipay et al [49]. They established that cartilage and bone formation in the developing limbs of a mouse embryo took place between E12.5 and E16.5. The first cartilaginous areas were identified in the forelimb at E12.5, while the mineralization-specific Alizarin red staining was recognizable from E15.5. Thus, we decided that E15 was the most suitable stage to examine the DNA methylation-related gene expression. At that stage, chondrocytes in mature hyaline cartilage could be easily identified, and the developmental stage of chondrocytes in E15 mouse embryos is almost the same as in the 6-day-old primary murine micromass cultures. The *in situ* hybridization results were in a good agreement with the findings of the quantitative real-time PCR studies. The most important similarity between the results of *in situ* hybridization and RT-qPCR was that in

both cases, *Tet1* had the strongest expression pattern among the three examined epigenetic regulators. The significant role of this epigenetic factor in osteochondrogenic differentiation was proved by the experiments in which the specific knockout of *Tet1* impaired the skeletal development of the mutant mice: the examined animals showed growth irregularities or even embryonic lethality [50]. Specific knockdown of *Tet1* in the ATDC5 chondroprogenitor cell line also altered the chondrogenic differentiation of the cells [13]. Based on our results and the experimental data mentioned earlier, it is reasonable to suggest that *Tet1* has a major role in cartilage formation.

We have also investigated the effects of the DNA methylation inhibitor 5-azaC on chondrocyte differentiation. Based on data found in earlier studies [33] [51] [52], we applied the compound at a final concentration of 10 μ M. This concentration was five-fold higher than that applied to mature chondrocytes by Duan et al [53], but it was well tolerable by the chondrogenic cells as shown by the viability assay. The cell proliferation assay indicated that 5-azaC affected two important phases of *in vitro* chondrogenesis: the proliferating phase between days 0 and 3 (with mostly chondroprogenitor cells and early chondroblasts present in the micromass culture), and also the differentiating phase between days 3 and 6 (with chondroblasts and mature chondrocytes that produce high amount of cartilage-specific ECM). We found that the early phase of chondrogenesis was more strongly affected by the treatment, causing extreme loss in the proliferative ability of the cells. The RT-qPCR results confirmed that 5-azaC was indeed modulating DNA methylation during *in vitro* chondrogenesis: the expression of *Dnmt3a* gene was significantly inhibited, while the expression of *Tet1* gene, which has a role in DNA demethylation, was not altered. 5-azaC treatment has been documented to enhance the chondrogenic differentiation of human MSCs *in vitro* [54]. We proved that 5-azaC had a differentiation state-dependent inhibitory mechanism during *in vitro* hyaline cartilage formation. The mRNA expression levels of *Sox9*, *Col2a1* and *Acan* significantly decreased when 5-azaC was applied during the early stages of chondrogenesis. However, when DNA methylation was inhibited during later stages of chondrogenesis, *Sox9* and *Acan* became significantly upregulated. These findings are in good correlation with the results obtained with DMMB staining, where the primary murine high density cultures treated during the early phase of chondrogenesis displayed a weaker metachromasia than those which received 5-azaC during the second phase of the culturing.

5. Conclusions

This is the first study to report the differentiation stage-dependent transcript expression levels of key enzymes mediating DNA methylation and demethylation during *in vitro* chondrogenesis of primary chondrifying micromass cultures. Both enzyme systems showed increased expression during the early stages of chondrogenic differentiation, which was followed by a gradual decrease at the later phase. The two epigenetic mechanisms (i.e., DNA methylation and demethylation) are mutually exclusive; however, considering different regulatory regions (i.e., promoters and enhancers), the two processes can also take place simultaneously. The differentially methylated regions must be identified in order to provide a better insight into chondrogenesis and discover genes that may serve as drug targets. The differentiations stage-dependent effects of 5-azaC on chondrogenic cells suggest the need of careful design for therapeutic application of this compound during the intended treatment of joint diseases. Moreover, 5-azaC can also inhibit RNA methylation, which might provide another regulatory layer for chondrogenic differentiation [55]. Therefore, it is reasonable to consider that option when the effect of 5-azaC treatment is evaluated.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Sequences of primer pairs used for the PCR assay analyses, Table S2: Sequences of primer pairs used for the RT-qPCR reactions, Table S3: Sequence data of the 3'UTR regions of *Dnmt3a*, *Ogt* and *Tet1* genes with insert flanking T7 promoters for antisense probe preparation.

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