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DNA Methylation-Based Regulation of Human Bone Marrow-Derived Mesenchymal Stem/Progenitor Cell Chondrogenic Differentiation

Yu Nomura[a] Emilio Satoshi Hara[b] Yuya Yoshioka[a] Há Thi Nguyen[a, c] Shuji Nosho[a] Taishi Komori[a] Kei Ishibashi[a, c] Toshitaka Oohashi[c] Mitsuaki Ono[c] Takuo Kuboki[a]

[a]Department of Oral Rehabilitation and Regenerative Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [b]Department of Biomaterials, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [c]Department of Molecular Biology and Biochemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Keywords
Mesenchymal stem cells · Stem cell differentiation · DNA methylation · DNA methyltransferases · 5-Azacitidine · Tissue engineering of cartilage and bone

Abstract
Stem cells have essential applications in in vitro tissue engineering or regenerative medicine. However, there is still a need to understand more deeply the mechanisms of stem cell differentiation and to optimize the methods to control stem cell function. In this study, we first investigated the activity of DNA methyltransferases (DNMTs) during chondrogenic differentiation of human bone marrow-derived mesenchymal stem/progenitor cells (hBMSCs) and found that DNMT3A and DNMT3B were markedly upregulated during hBMSC chondrogenic differentiation. In an attempt to understand the effect of DNMT3A and DNMT3B on the chondrogenic differentiation of hBMSCs, we transiently transfected the cells with expression vectors for the two enzymes. Interestingly, DNMT3A overexpression strongly enhanced the chondrogenesis of hBMSCs, by increasing the gene expression of the mature chondrocyte marker, collagen type II, more than 200-fold. Analysis of the methylation condition in the cells revealed that DNMT3A and DNMT3B methylated the promoter sequence of early stem cell markers, *NANOG* and *POU5F1* (OCT-4). Conversely, the suppression of chondrogenic differentiation and the increase in stem cell markers of hBMSCs were obtained by chemical stimulation with the demethylating agent, 5-azacitidine. Loss-of-function assays with siRNAs targeting DNMT3A also significantly suppressed the chondrogenic differentiation of hBMSCs. Together, these results not only show the critical roles of DNMTs in regulating the chondrogenic differentiation of hBMSCs, but also suggest that manipulation of DNMT activity can be important tools to enhance the differentiation of hBMSCs towards chondrogenesis for potential application in cartilage tissue engineering or cartilage regeneration.

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E-Mail karger@karger.com
www.karger.com/cto

Emilio Satoshi Hara, DDS, PhD
Department of Biomaterials, Okayama University Graduate School of Medicine Dentistry and Pharmaceutical Sciences
2-5-1 Shikata-cho, Okayama 700-8525 (Japan)
E-Mail gmd421209@s.okayama-u.ac.jp

Mitsuaki Ono
Molecular Biology and Biochemistry, Okayama University Graduate School of Medicine Dentistry and Pharmaceutical Sciences
2-5-1 Shikata-cho, Okayama 700-8525 (Japan)
E-Mail mitsuaki@md.okayama-u.ac.jp

Introduction

Stem cells are characterized by their ability to self-renew and capacity to differentiate into several other cell types [Jaenisch and Young, 2008; Tewary et al., 2018]. Identification and characterization of adult stem cells in various tissues have led to a greater understanding of tissue development, homeostasis, and regeneration [Lin et al., 2019]. A deeper understanding of the biology of stem cells is still required for the development of novel methods to control stem cell functions by intracellular or extracellular factors, and for further improvement in the efficacy of methods for regenerative medicine [Tewary et al., 2018].

Most of the cellular functions are initially regulated by the gene transcription pattern, which involves a complex biological process regulated not only by the genetic code, but also by epigenetic mechanisms. DNA methylation and histone modifications are the most important epigenetic regulations of DNA. In particular, DNA methylation involves the addition of a methyl group to the carbon-5 position in the pyrimidine ring of the cytosines nucleotides [Bird, 1986; Perez-Campo and Riancho, 2015]. It is known that methylation of the cytosine-phosphate-guanine (CpG) islands located on the promoter regions, or the first exon, inhibits the transcription of the corresponding genes [Bird, 1986; Denis et al., 2011]. Therefore, DNA methylation determines the global gene expression pattern, and directly controls stem cell differentiation during tissue development or regeneration, and is also associated with a number of key biological processes, including aging and cancer [Berdasco and Esteller, 2011; Challen et al., 2011; Denis et al., 2011; Feng et al., 2014; Perez-Campo and Riancho, 2015].

Methylation of CpGs is catalyzed by a family of DNA methyltransferases (DNMTs), consisting basically of 3 members, i.e., DNMT1, DNMT3A, and DNMT3B. Gene deletion of DNMTs results in embryo mortality [Li et al., 1992; Okano et al., 1999], indicating that DNA methylation is essential for normal embryonic development by epigenetic modification of gene expression in a tissue- and context-specific manner [Perez-Campo and Riancho, 2015]. DNMT1 has a preference for hemi-methylated DNA (one strand), and therefore is mainly involved in the maintenance of pre-existing methylation, and is important for transferring patterns of methylation to a newly synthesized strand after DNA replication [Goll and Bestor, 2005]. DNMT3A and DNMT3B act as de novo methyltransferases, modifying unmethylated DNA [Okano et al., 1999; Goll and Bestor, 2005]. During embryonic development, the two major de novo methyltransferases, DNMT3A and DNMT3B, are known to silence stem cell marker genes (e.g., *NANOG*, *POU5F1*/OCT-4) [Li et al., 2007], as well as cell type-specific genes required for the establishment of genomic methylation patterns [Okano et al., 1999].

During embryonic development, mesenchymal stem/progenitor cells (MSCs) condensate and differentiate into chondrocytes to form the limb buds [Shimizu et al., 2007]. These chondrocytes proliferate and differentiate into mature chondrocytes, eventually forming the entire limb [Shimizu et al., 2007]. Understanding the methylation condition during the process of MSC-to-chondrocyte differentiation may enable the development of novel techniques for application in cartilage regeneration.

Previous studies have shown that the DNA methylation levels of CpG-rich promoters of chondrocyte-related genes (e.g., SOX9) are kept hypomethylated during chondrogenesis of human MSCs [Ezura et al., 2009; Herlofsen et al., 2013]. Although these studies evaluated the methylation of chondrocyte-related genes, the expression levels of DNMTs and their roles in regulating the expression of stem cell marker genes (e.g., *POU5F1*, *NANOG*) during chondrogenic differentiation of MSCs are still unknown. In this study, we first investigated the DNA methylation patterns during chondrogenic differentiation of human bone marrow-derived MSCs (hBMSCs), analyzed the methylation condition of stem cell markers, and finally manipulated the chondrogenic differentiation of hBMSCs by exogenous stimulation with demethylating agent, 5-azacitidine (5-Aza), or by inducing overexpression of DNMT3A and DNMT3B in the cells. The results showed that the expression levels of both DNMT3A and DNMT3B, but not DNMT1, are increased during chondrogenic differentiation of hBMSCs. DNMT3A and DNMT3B suppressed *NANOG* and *POU5F1* expression, and particularly DNMT3A had a remarkable role in promoting chondrogenesis of hBMSCs.

Materials and Methods

Cells, Culture Conditions, and Chondrogenic Differentiation

hBMSCs were purchased from Lonza (Walkersville, MD, USA) and cultured in alpha-Modified Eagle Medium (α-MEM, Invitrogen, Carlsbad, CA, USA) containing 15% FBS (Invitrogen), 100 mM L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries, Osaka, Japan), 1% penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 1% L-glutamine (Invitrogen). In the experiments, cells from at least 2 individuals and from the third to the sixth passage were used.

Table 1. List of primer pairs used for real-time RT-PCR analysis

Gene name (accession No.)	Direction	Nucleotide sequence
S29 (BC032813)	Sense	5′-TCTCGCTCTTGTCGTGTCTGTTC-3′
	Anti-sense	5′-ACACTGGCGGCACATATTGAGG-3′
NANOG (NM_024865.2)	Sense	5′-GCCTTCACACCATTGCTAT-3′
	Anti-sense	5′-TCTCCAACATCCTGAACCT-3′
POU5F1 (NM_001159542.1)	Sense	5′-GAAAGGGACCGAGGAGTA-3′
	Anti-sense	5′-CCGAGTGTGGTTCTGTAAC-3′
DNMT3A (NM_175629.2)	Sense	5′-GCAGCCATTAAGGAAGAC-3′
	Anti-sense	5′-TGGTTATTAGCGAAGAACATC-3′
DNMT3B (NM_006892.3)	Sense	5′-TTACCTTACCATCGACCTCACA-3′
	Anti-sense	5′-CTGTCTCCATCTCCACTGTCT-3′
DNMT1 (NM_001379.2)	Sense	5′-CCATCAGGCATTCTACCA-3′
	Anti-sense	5′-CGTTCTCCTTGTCTTCTCT-3′
COL10A1 (NM_000493)	Sense	5′-GAATGCCTGTGTCTGCTT-3′
	Anti-sense	5′-TCATAATGCTGTTGCCTGTT-3′
COL2A1 (NM_00184)	Sense	5′-TGGAGCAGCAAGAGCAAGGAGAAG-3′
	Anti-sense	5′-CCGTGGACAGCAGGCGTAGG-3′
ACAN (BC036445)	Sense	5′-GGCATTTCAGCGGTTCCTTCTCC-3′
	Anti-sense	5′-CAGCAGTTGTCTCCTCTTCTACGG-3′
NT5E (BC065937)	Sense	5′-TCCTTGCCTTTAATGTGTGAA-3′
	Anti-sense	5′-GTTGCTGACCCTGAGTAATC-3′
THUY1 (BC065559)	Sense	5′-GGACCTGATGGAGAGTGAGA-3′
	Anti-sense	5′-CCGATGGGCAAGGATGAC-3′
ENG (BC014271)	Sense	5′-TGAACTTGCCTAACTAACTGG-3′
	Anti-sense	5′-ATTGGTGGTGAATACACAGG-3′

For chondrogenic differentiation, hBMSCs were cultured in micromasses or pellets, as reported previously [Hara et al., 2015]. In brief, for micromass culture, cells were trypsinized and collected by centrifugation, resuspended at a concentration of 2×10^7 cells/mL, and cultured in 10-μL micromasses. For pellet culture, hBMSCs were trypsinized, centrifuged, counted, and aliquoted in 15-mL tubes at a concentration of 1×10^6 cells per 500 μL of culture medium. The cell-containing tubes were then centrifuged to make the cell pellets. Chondrogenic medium consisted of DMEM (low glucose, Invitrogen) containing 6 μg/mL of insulin, transferrin, and selenous acid (ITS solution, BD, Bedford, MA, USA), L-ascorbic acid (50 μg/mL), TGF-β3 (5 ng/mL, R&D Systems, Minneapolis, MN, USA), fluocinolone acetonide (100 nM, Tokyo Chemical Industry Inc., Tokyo, Japan), and antibiotics, as reported [Hara et al., 2015, 2016]. Cultures were maintained for 21 days, with the medium changed every 3–4 days, and further submitted to analysis of mRNA levels or histology.

For chemical control of hBMSC function, 5-Aza (Sigma-Aldrich) was added to the hBMSC culture for 24 and 48 h. Undifferentiated hBMSCs were then washed and collected for total RNA purification, and further analysis of the expression of stem cell marker genes. Alternatively, hBMSCs were trypsinized, collected by centrifugation, and cultured in micromasses for evaluation of chondrogenic differentiation after 5-Aza treatment.

Histological and Immunohistochemical Analysis

hBMSC micromasses/pellets were cultured for 21 days in chondrogenic medium, and then fixed with 4% paraformaldehyde, dehydrated through immersion in increasing concentrations of ethanol (50, 70, 80, 90, and 100%), and embedded in paraffin. Sections of 5 μm were cut using a microtome (Thermo Scientific, HM 355S Automatic Microtome), hydrated, and stained with toluidine blue or safranin O for glycosaminoglycans. Sections were also used for immunohistochemical analysis of collagen type II expression in the pellets using a specific rabbit polyclonal antibody (Chemicon, Billerica, MA, USA).

Reverse Transcription and Real-Time Reverse Transcription-Polymerase Chain Reaction

Total cellular RNA was extracted using Purelink (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's instructions. hBMSCs, either undifferentiated or differentiat-

Table 2. List of primer pairs used for methylation-specific PCR analysis

Gene	Species	M or UM	Primer sequence	PCR product length, bp
NANOG	Human	M	5′ -TTTGGGTAATATGGTGAAATTTTGT-3′ (S) 5′ -TACCAAACCGAAATACAATAACGTA-3′ (AS)	162
		UM	5′ -TTTGGGTAATATGGTGAAATTTTGT-3′ (S) 5′ -TACCAAACCAAAATACAATAACATA-3′ (AS)	162
POU5F1	Human	M	5′ -TTTAGGAAGTTGAGGTAGGAGAATC-3′ (S) 5′ -AAAATTACAAATATAAACCACCGCA-3′ (AS)	169
		UM	5′ -TTTAGGAAGTTGAGGTAGGAGAATT-3′ (S) 5′ -AAAATTACAAATATAAACCACCACA-3′ (AS)	169

M, methylated-specific primer; UM, unmethylated-specific primer; S, sense; AS, antisense.

ed (in pellets or micromasses), were washed with PBS and lysed with lysing buffer before total cellular RNA purification. To remove potential residual DNA, the samples were treated with DNase I (DNASE I, Invitrogen). RNA samples were reverse-transcribed by using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. A real-time RT-PCR method was used for mRNA quantitation using the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) and Chromo4™ real-time detector (Bio-Rad), as described previously. The ΔΔCt method was used for calculation of the relative gene expression values. The levels of mRNA of interest were normalized to that of the reference gene, ribosomal protein *S29*. The primer sequences are shown in Table 1.

Transient Transfections and Inhibition Assays

For transfection experiments, hBMSCs were seeded in 10-cm dishes and cultured until a subconfluent condition was reached. hBMSCs were then transiently transfected with DNMT3A and DNMT3B overexpression vectors with Lipofectamine 3000 (Life Technologies), or with scrambled siRNA (control) or siRNA targeting DNMT3A with RNAiMAX (Life Technologies), according to the manufacturer's instructions. Details of the plasmid constructs were published previously [Bachman et al., 2001; Jair et al., 2006]. The sequences were inserted in a pcDNA3 backbone, and the empty vector was used as a control in the transfection assays. Three hours (for *POU5F1* and *NANOG* mRNA levels) and 12 h (for *DNMT3A* or *DNMT3B* mRNA levels) after transfection, hBMSCs were washed, and total RNA was collected for gene expression analysis. Alternatively, hBMSCs were trypsinized, collected by centrifugation and cultured in micromass or pellet cultures for evaluation of chondrogenic differentiation.

In silico Target Prediction of Methylation Sites

In silico analysis of methylation sites in the early stem cell markers, *POU5F1* and *NANOG*, was performed with the online software MethPrimer (http://www.urogene.org/methprimer) for CpG islands, set for ≥70% of CG%, 60% of the observed/expected CpG ratio, and a minimum of 200 bp. The promoter sequence was obtained from the eukaryotic promoter database (https://epd.epfl.ch), from 0 to 4,000 bp relative to the transcription starting site.

Methylation Assay

Bisulfite conversion of DNA was performed by using the EpiTect Fast Bisulfite Conversion Kit (Qiagen, Hilden, German). Thermal cycler conditions for bisulfite conversion were two repeated cycles of denaturation (5 min, 95 °C) and incubation (10 min, 60 °C), as recommended by the manufacturer. The DNA methylation pattern was analyzed by methylation-specific PCR (EpiTect MSP, Qiagen) using GoTaq DNA Polymerase (Promega, Madison, WI, USA) and unmethylated-specific and methylated-specific primers (Table 2) designed with MethPrimer online software. The thermal cycler conditions were initial denaturation (5 min, 95 °C), and 35 cycles of denaturation (1 min, 95 °C), annealing (20 s, 57 °C), extension (20 s, 72 °C), followed by final extension (7 min, 70 °C). PCR products (20 μL) were electrophoresed in agarose gel (1%), and the bands were detected under ultraviolet light and photographed with an image detector and analyzer (LAS 4000 mini, Fujifilm, Tokyo, Japan).

Statistical Analysis

Analysis of the differences between groups was performed with the unpaired Student *t* test, or one-way ANOVA followed by a Tukey post hoc correction test when appropriate. GraphPad Prism 7 software was used for the analyses.

Results

Expression Pattern of DNA Methyltransferases during hBMSC Chondrogenic Differentiation

First, hBMSCs were cultured in micromasses and induced to differentiate into chondrocytes, which was determined by safranin O staining for mature chondrocyte-

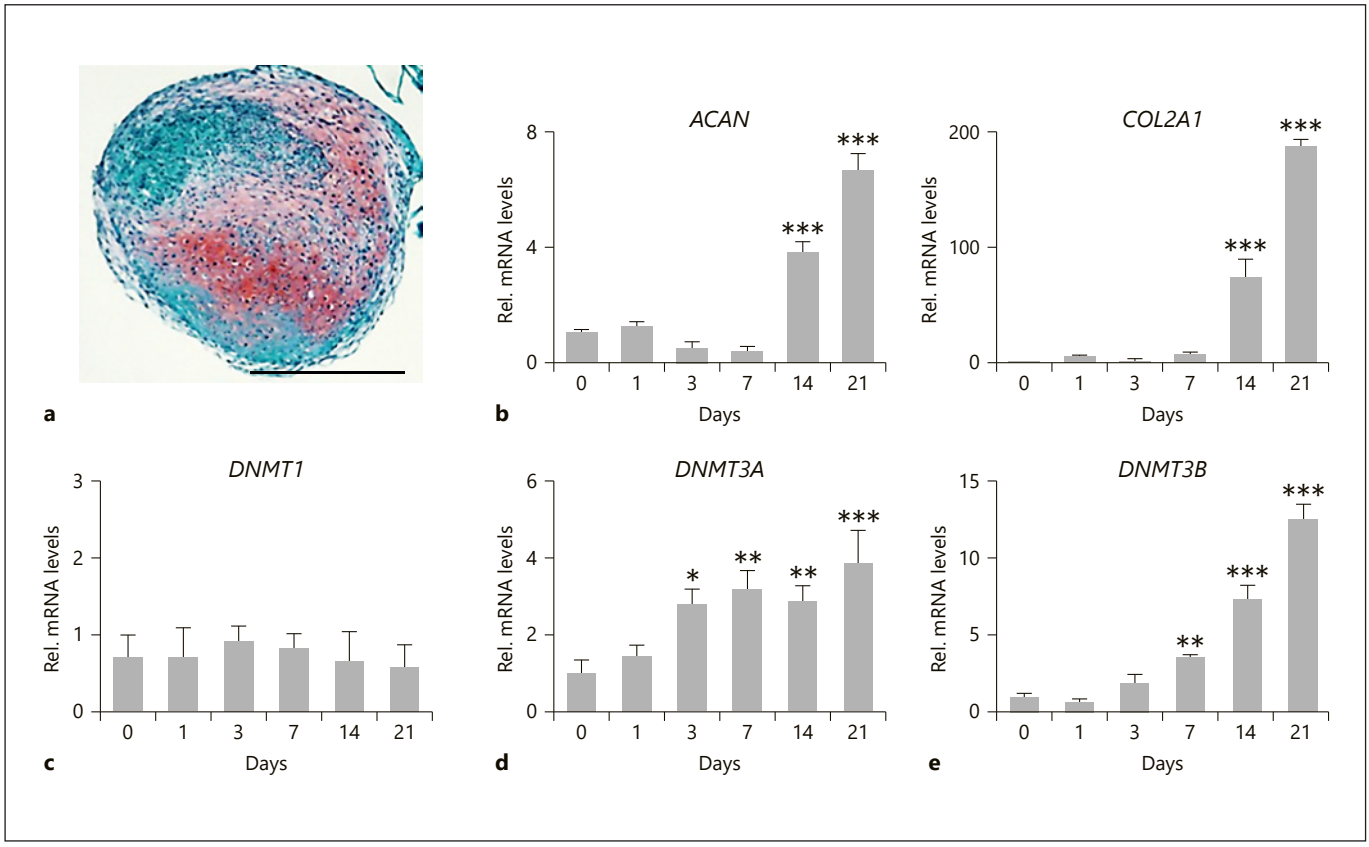

Fig. 1. a Safranin O staining of hBMSCs cultured in micromasses. Note the deposition of glycosaminoglycans (red area) stained with safranin O. Scale bar, 500 μm. **b** Chondrogenesis of hBMSCs was also confirmed by the increase in mRNA levels of the two major chondrocyte marker genes, *ACAN* and *COL2A1*. Note the significant increase in the mRNA levels of the two genes from day 14 of culture onwards. Gene expression levels of *DNMT1* (**c**), *DNMT3A* (**d**), and *DNMT3B* (**e**) during the 21-day period of chondrogenic differentiation. Note that *DNMT1* mRNA levels did not change throughout the entire period. *DNMT3A* mRNA levels increased significantly from day 3 onwards, reaching a 3-fold relative increase compared to day 0. *DNMT3B* mRNA levels increased dramatically from day 7 onwards, reaching a peak of a more than 10-fold relative increase on day 21. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA/Tukey test.

derived glycosaminoglycans (Fig. 1a), as well as by the increase in the expression of mature chondrocyte marker genes, aggrecan (*ACAN*) and collagen type II (*COL2A1*) from day 14 onwards (Fig. 1b). We then investigated the expression pattern of DNMT1, DNMT3A, and DNMT3B throughout the entire period of chondrogenic differentiation of hBMSCs. As shown in Figure 1c–e, there were no significant changes in the expression of DNMT1, but the expression levels of DNMT3A and DNMT3B significantly increased from day 3 onwards, with a substantial increase of almost 10 times in DNMT3B expression levels after day 14. These results indicate that DNMT1 is not directly associated with the expression of the genes related to chondrogenic differentiation. On the other hand, the data suggested that DNMT3A and DNMT3B could be the major DNA methyltransferases associated with chondrogenic differentiation of hBMSCs.

Manipulation of Chondrogenic Differentiation of hBMSCs Using Overexpression Vectors

To evaluate the direct effects of DNMT3A and DNMT3B on chondrogenesis, hBMSCs were transiently transfected with plasmid vectors inducing overexpression of the two DNA methyltransferases (Fig. 2a). Surprisingly, DNMT3A overexpression strongly enhanced the chondrogenic differentiation of hBMSCs, as demonstrated by intense toluidine blue staining for glycosaminoglycans and immunostaining for collagen type II

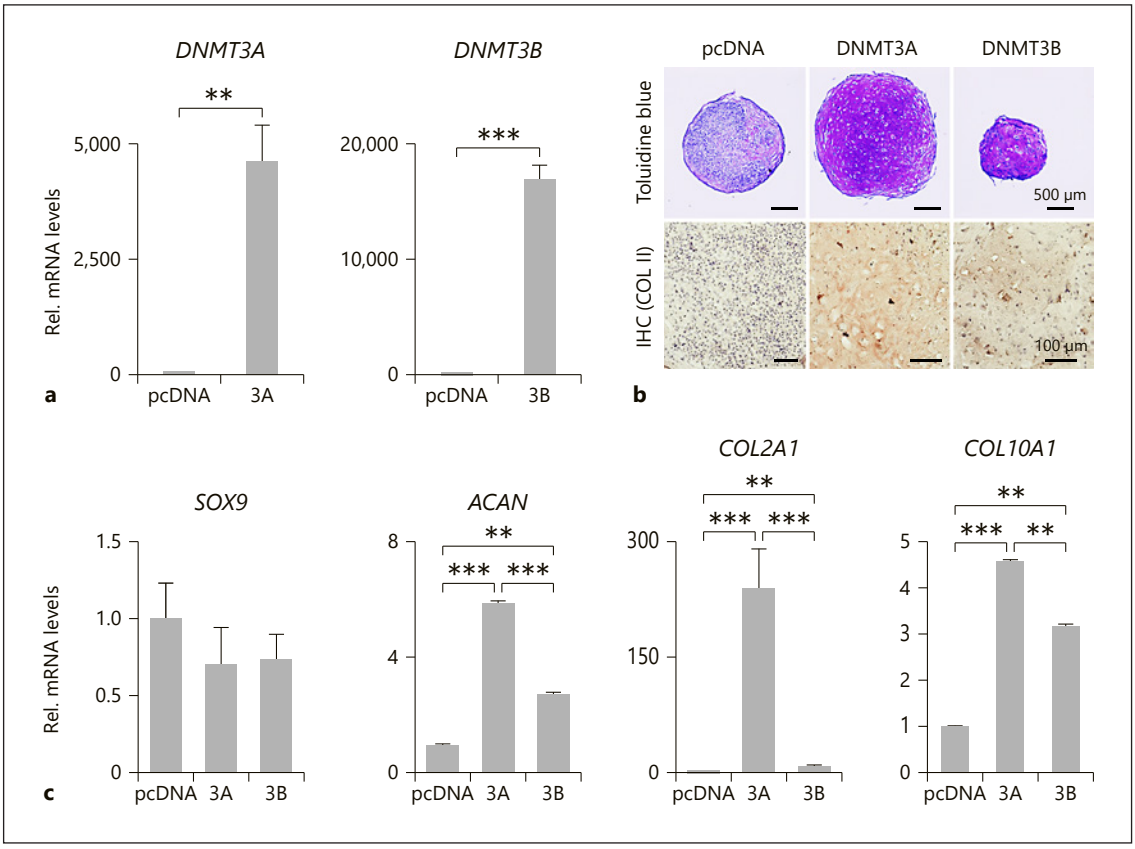

Fig. 2. a Relative mRNA levels of *DNMT3A* and *DNMT3B* in hBMSCs, 12 h after the transfection of overexpression vectors. Note the dramatic increase in the mRNA levels of *DNMT3A* and *DNMT3B* in the cells. **b** Toluidine blue staining and immunostaining for collagen type II (COL II) in the histological sections of hBMSC micromasses after 21 days of culture. Note the remarkable deposition of glycosaminoglycans and collagen type II by the cells transfected with the DNMT3A overexpression vector. DNMT3B overexpression also increased cartilaginous matrix deposition, but to a lesser extent compared to DNMT3A. **c** Gene expression levels of the early chondrocyte marker, *SOX9*, as well as of the mature chondrocyte markers, *ACAN*, *COL2A1*, and *COL10A1*, after overexpression of DNMTs. Note that overexpression of either *DNMT3A* or *DNMT3B* did not affect *SOX9* mRNA levels, but markedly increased the expression levels of mature chondrocyte marker genes. 3A, DNMT3A; 3B, DNMT3B. SOX9 mRNA levels were analyzed 1 day after the start of chondrogenic differentiation. *ACAN*, *COL2A1*, and *COL10A1* mRNA levels were analyzed on the 21st day of chondrogenic differentiation. ** $p < 0.01$, *** $p < 0.001$, ANOVA/Tukey test. Transfection of empty vector (pcDNA) was used as a control.

(Fig. 2b). The gene expression analysis of *ACAN* and *COL2A1* further confirmed the dramatic effect of DNMT3A overexpression on chondrogenesis of hBMSCs. On the other hand, DNMT3B also enhanced the chondrogenic differentiation of hBMSCs, however to a lesser extent compared to DNMT3A, as shown by the histological analysis, and more clearly by the analysis of mRNA expression levels of *ACAN* and *COL2A1* (Fig. 2c).

We also analyzed the expression levels of the master regulator of chondrogenesis, SOX9, 1 and 3 days after transfection with DNMT3A and DNMT3B. Interestingly, however, there was no significant difference in the expression levels of SOX9, either at mRNA levels (Fig. 2c) or at protein levels (data not shown). These results indicate that DNMT3A and DNMT3B could not be directly promoting methylation of the genes associated with chondrocyte differentiation (*SOX9*, *ACAN*, *COL2A1*), but, on the other hand, the methyltransferases could be promoting methylation in the promoter region of genes associated with the maintenance of the stem cell phenotype of hBMSCs.

Methylation Pattern in the Promoter Sequence of Stem Cell Marker Genes

Next, we investigated the methylation condition of the early stem cell markers, *POU5F1* (OCT-4) and *NANOG*, as well as other markers for MSCs, including ecto-5′-

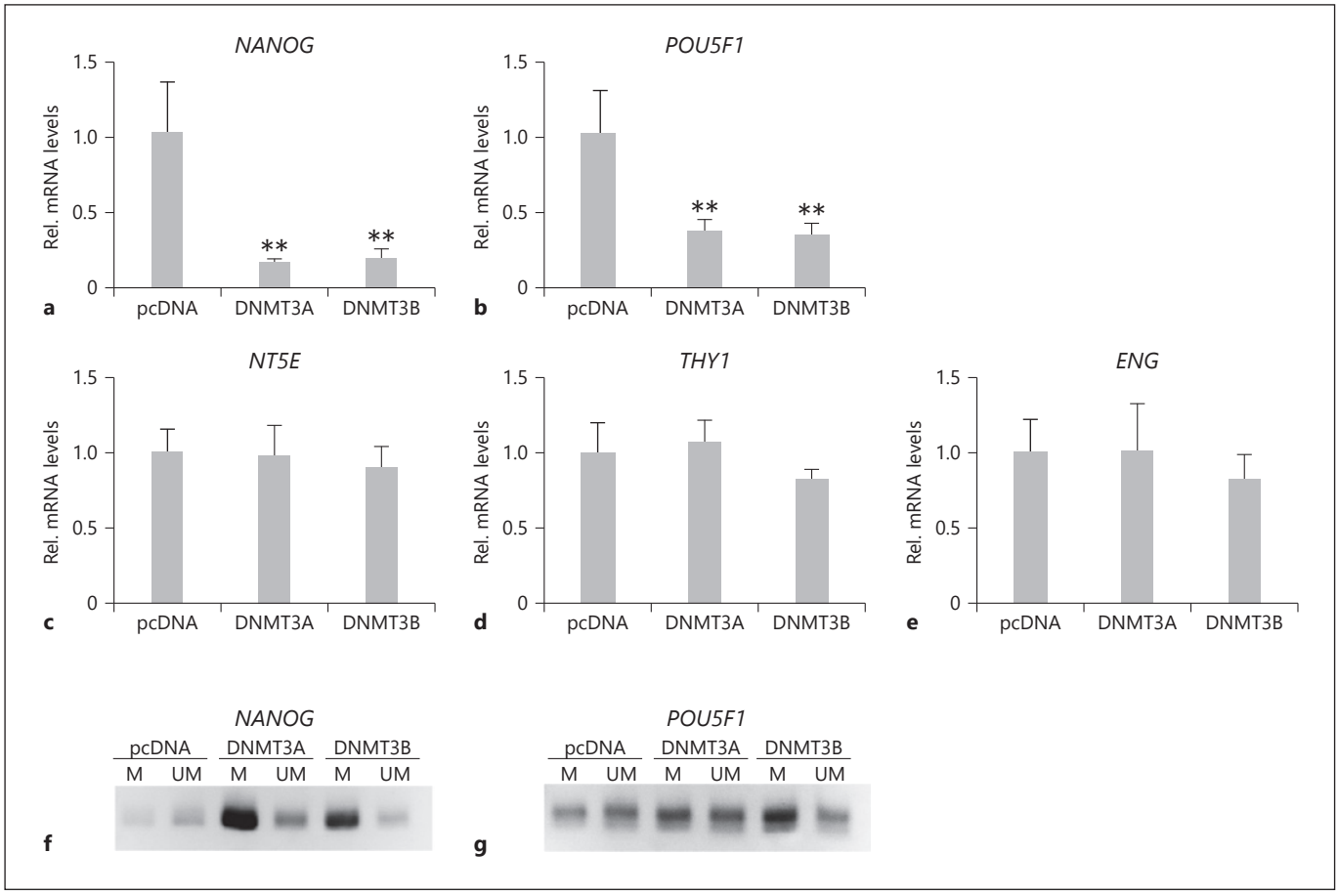

Fig. 3. Relative mRNA levels of *NANOG* (**a**), *POU5F1* (OCT-4; **b**), *NT5E* (CD73; **c**), *THY1* (CD90; **d**), and *ENG* (CD105; **e**) following overexpression of DNMT3A and DNMT3B. Note the significant suppression of *NANOG* and *POU5F1* expression levels, whereas those of *NT5E*, *THY1*, and *ENG* were not affected by DNMT3A or DNMT3B overexpression. mRNA levels of *POU5F1* and *NANOG* were analyzed 3 h posttransfection. **f, g** Results of methylation-specific PCR showing CpG methylation of promoter regions of *NANOG* and *POU5F1* after overexpression of DNMT3A or DNMT3B. ** $p < 0.01$, ANOVA/Tukey test. Transfection of empty vector (pcDNA) was used as a control. M, methylated; UM, unmethylated.

nucleotidase (*NT5E*, CD73), *THY1* (CD90), and endoglin (*ENG*, CD105). As shown in Figure 3, overexpression of DNMT3A and DNMT3B strongly suppressed the gene transcription of *NANOG* and *POU5F1*, but no changes in the gene expression of NT5E, THY1, and ENG could be observed. Since a previous study showed that DNMT3A and DNMT3B promote methylation of *Pou5f1* and *Nanog* in stem cells [Li et al., 2007], we also analyzed the methylation state of these two genes upon overexpression of DNMT3A and DNMT3B. The results revealed that both DNMT3A and DNMT3B were able to promote the methylation of *NANOG* promoter. On the other hand, *POU5F1* methylation was only observed by DNMT3B overexpression. These results in part explain the decrease in mRNA transcription levels of the two stem cell markers, *POU5F1* and *NANOG*.

Chemical Manipulation of hBMSC Stem Cell Phenotype and Chondrogenic Differentiation

Finally, hBMSCs were chemically stimulated with an exogenous demethylating agent, 5-Aza. As shown in Figure 4a and b, stimulation with 5-Aza induced a significant decrease in the expression levels of *DNMT3A*, but not in that of *DNMT3B*. Additionally, 5-Aza significantly increased the expression levels of the stem cell markers *POU5F1* and *NANOG* (Fig. 4c, d). In accordance with the overexpression experiments, 5-Aza treatment did not induce any change in the mRNA transcription levels of the

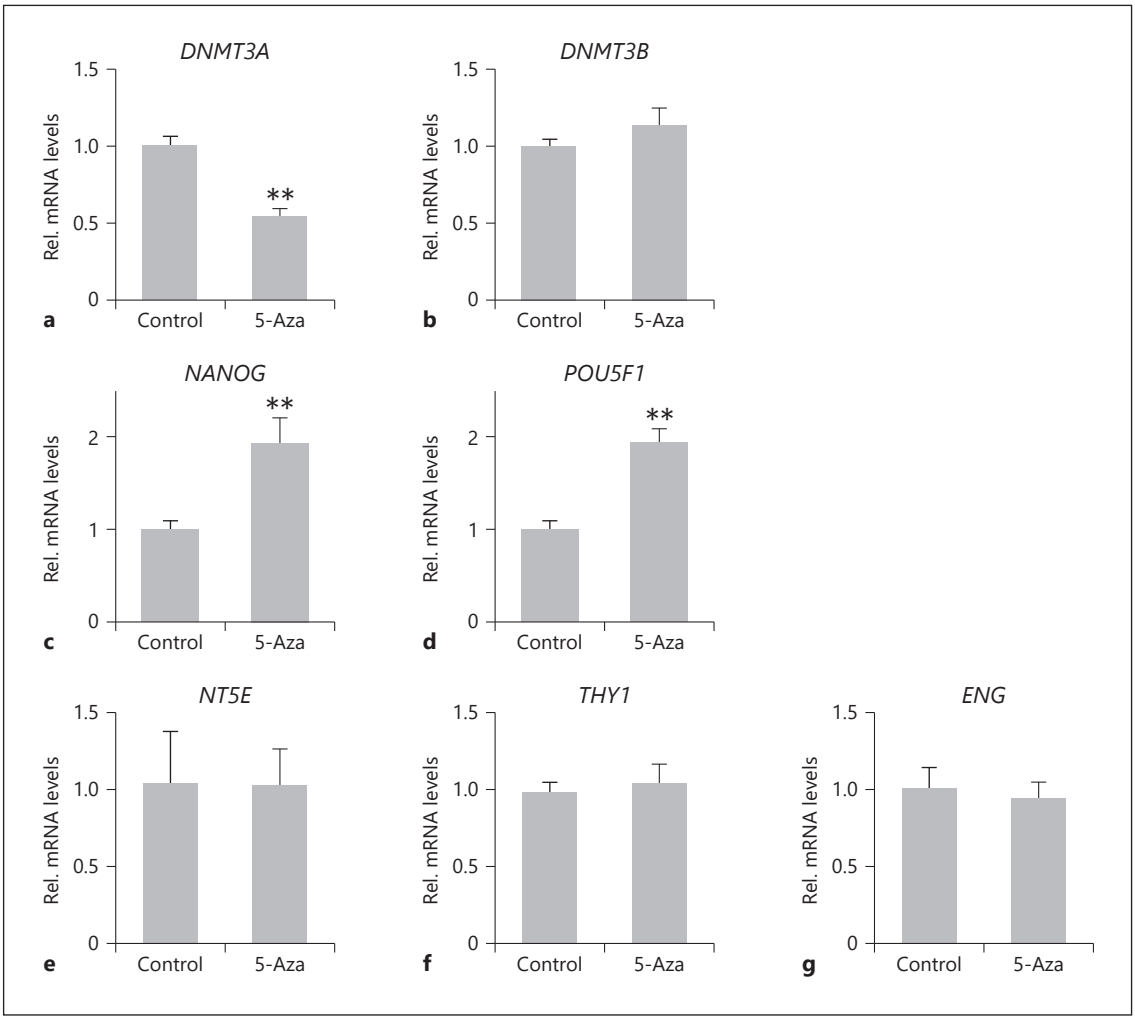

Fig. 4. a, **b** Relative mRNA levels of *DNMT3A* and *DNMT3B* in hBMSCs upon treatment with 5-Aza. Note a significant inhibition of *DNMT3A* by 5-Aza stimulation. **c–g** Relative mRNA levels of *NANOG*, *POU5F1*, *NT5E*, *THY1*, and *ENG* in hBMSCs upon treatment with 5-Aza. Note a significant increase in the gene expression of *NANOG*, *POU5F1*, but not *NT5E*, *THY1*, and *ENG* after 5-Aza treatment. mRNA levels of these stem cell markers were analyzed 24 h poststimulation. ** $p < 0.01$, ANOVA/Tukey test.

other analyzed markers for MSCs, *NT5E*, *THY1*, and *ENG* (Fig. 4e–g). Next, we attempted to control chondrogenic differentiation with 5-Aza, and indeed 5-Aza markedly suppressed chondrogenesis of hBMSCs, as demonstrated by toluidine blue staining of hBMSC micromass cultures, and mRNA levels of *ACAN*, *COL2A1*, and *COL10A1* (Fig. 5). Together, these results suggested that 5-Aza could be affecting the transcription of specific genes associated with undifferentiated hBMSCs (i.e., *POU5F1*, *NANOG*) or differentiated hBMSCs (i.e., *COL2A1*, *ACAN*) in a direct and indirect manner, through a possible parallel regulation of DNMT expression.

Finally, to confirm the direct role of DNMT3A on the chondrogenesis of hBMSCs, we performed inhibition of DNMT3A by using a specific siRNA. As shown in Figure 6a, siDNMT3A significantly suppressed *DNMT3A* expression, and markedly inhibited chondrogenesis of hBMSCs, as demonstrated by toluidine blue staining and mRNA levels of *ACAN*, *COL2A1*, and *COL10A1* (Fig. 6b, c). Taken together, these data show that DNMTs play crucial roles in the control of genes associated with stemness of stem cells, which could further affect the chondrogenic differentiation of hBMSCs.

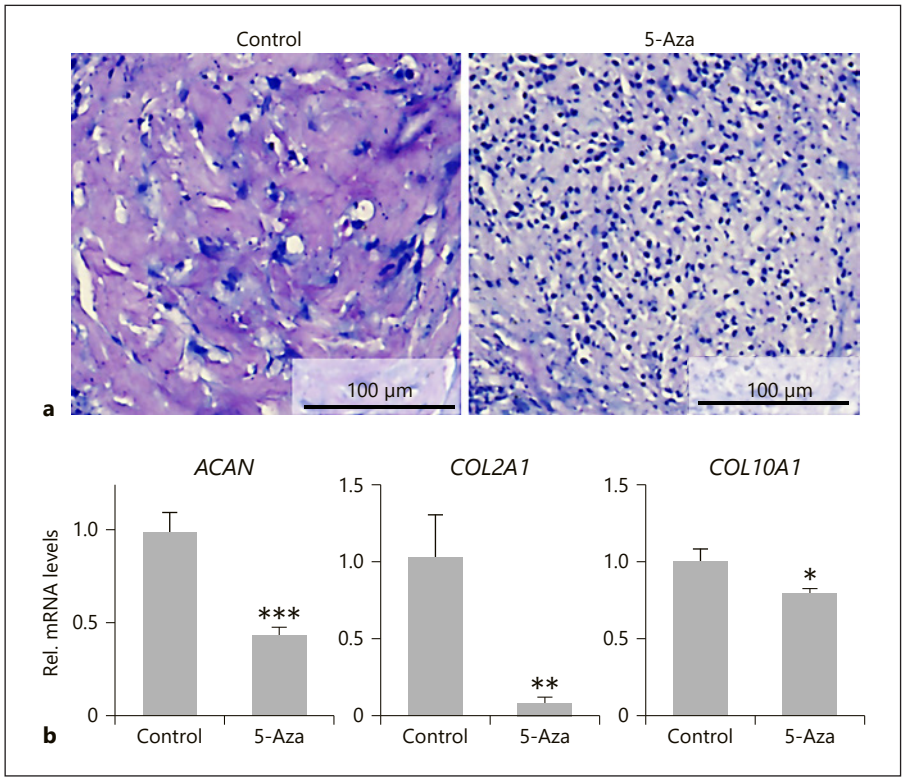

Fig. 5. Toluidine blue staining for glycosaminoglycans (**a**) and relative mRNA levels of *ACAN*, *COL2A1*, and *COL10A1* (**b**) after 21 days of chondrogenic differentiation of hBMSCs treated with 5-Aza. Note the inhibition in the glycosaminoglycan synthesis and the expression of chondrocyte marker genes by 5-Aza treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA/Tukey test.

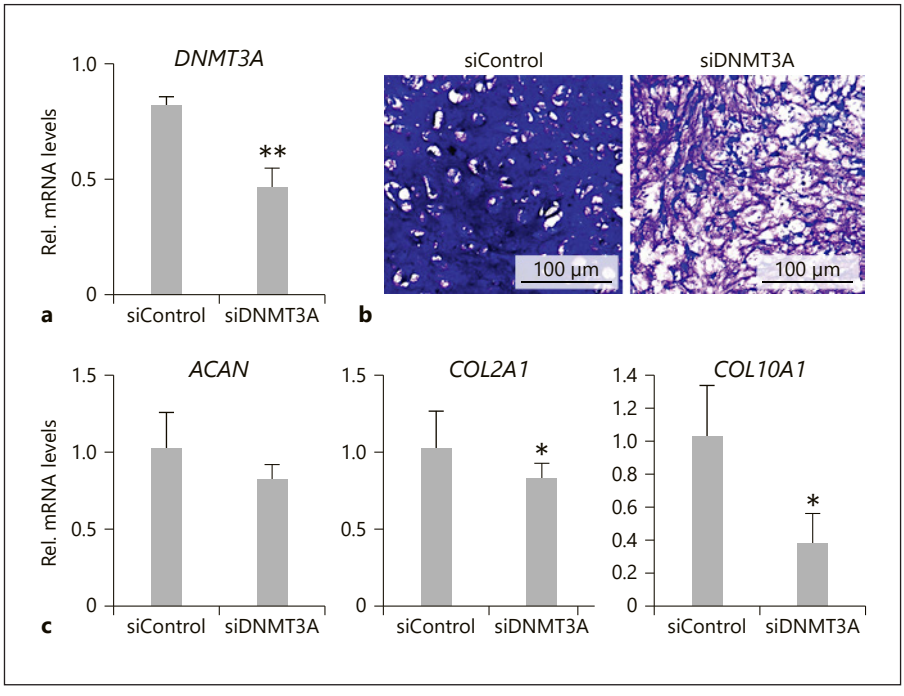

Fig. 6. **a** Relative mRNA levels of DNMT3A after inhibition with siRNA specific for *DNMT3A*. Toluidine blue staining for glycosaminoglycans (**b**) and relative mRNA expression levels of *ACAN*, *COL2A1*, and *COL10A1* (**c**) on day 21 of chondrogenic differentiation of hBMSC micromass cultures. Note the inhibition in the glycosaminoglycan synthesis and the expression of chondrocyte marker genes by siRNA inhibition. Although not statistically significant, there was a tendency in the suppression of *ACAN* mRNA levels after blockade of DNMT3A function with siDNMT3A. * $p < 0.05$, ** $p < 0.01$, ANOVA/Tukey test. Transfection of scrambled siRNA was used as a control.

Discussion

MSCs have been regarded as a high potential cell source in regenerative medicine. In particular, the chondrogenic differentiation of MSCs has been largely investigated. For instance, previous reports have identified the key factors that drive the chondrogenesis of MSCs [Furumatsu et al., 2005; Jin et al., 2007; Kita et al., 2008; Hara et al., 2015, 2016]. Additionally, other chemical drugs or compounds have been shown to actively induce chondrogenesis [Johnson et al., 2012; Hara et al., 2013]. Manipulation of MSC chondrogenic differentiation with biomaterials have also been extensively studied [Holland et al., 2005; Zhang et al., 2015; Tan and Hung, 2017]. However, there is still a need to understand the intracellular mechanisms regulating the chondrogenic differentiation of stem cells, which may allow further improvement in the methods for cell differentiation for application in tissue engineering or regenerative medicine.

A recent genome-wide map of quantified epigenetic changes after 7 days of chondrogenic differentiation of MSCs showed that the promoters of signature genes were hypomethylated both before and after chondrogenesis, suggesting that DNA methylation was not crucial in regulating transcription of these key genes during chondrogenesis [Herlofsen et al., 2013]. In this present study, however, we investigated the DNA methylation patterns during chondrogenic differentiation of hBMSCs, and demonstrated that the expression levels of both *DNMT3A* and *DNMT3B*, but not *DNMT1*, were increased during the 21-day differentiation process. We also demonstrated that DNMT3A in particular strongly enhanced the chondrogenesis of hBMSCs. Nevertheless, these findings could only be clarified by inducing overexpression of DNMT3A and DNMT3B. A possible reason for the apparent discrepancy between our study and the genome-wide analysis showing no marked difference in the methylation pattern of signature genes in MSCs and differentiated chondrocytes could be associated with a more complex regulatory mechanism of DNMT3A and DNMT3B expression in the physiological state [Denis et al., 2011; Jia et al., 2016].

Regarding the intracellular machinery of how DNMT3A and DNMT3B could be enhancing chondrogenesis, a possible mechanism would be by suppressing the expression of stem cell markers, *POU5F1* and *NANOG*. The effect of Dnmt3A and Dnmt3B in regulating the expression of *Nanog* and *Pou5f1* has been already clarified [Li et al., 2007]. Dnmt3A and Dnmt3B were shown to form a common complex to silence *Nanog* and *Pou5f1* transcription [Li et al., 2007].

On the other hand, by inhibiting the activity of DNMTs with 5-Aza, we could maintain the expression of stem cell markers. More interestingly, 5-Aza was shown to increase the mRNA levels of the early stem cell marker genes, *Pou5f1*, *Nanog*, and *Sox2*, and to convert somatic cells into tissue-regenerative MSCs [Tsuji-Takayama et al., 2004; Chandrakanthan et al., 2016]. Together, these data indicate that DNMTs play crucial roles both in the maintenance of the stemness as well as in the chondrogenic differentiation of MSCs.

Regarding the CpG methylation of chondrocyte marker genes, it has been extensively studied in cases of cartilage degenerative diseases, such as osteoarthritis [Barter et al., 2012; Kim et al., 2013]. Previous reports showed an increased DNA methylation in the *SOX9* promoter in damaged chondrocytes of OA patients compared to that of normal chondrocytes, suggesting that DNMT-associated downregulation of SOX9, which controls the gene expression of the main cartilage matrix components (i.e., collagen type II and glycosaminoglycans), could be leading to cartilage degeneration [Kim et al., 2013]. On the other hand, DNA methylation of the major markers of mature chondrocytes, in particular that of *ACAN*, was shown not to correlate with their mRNA transcription levels [Fernandez et al., 1985; Pöschl et al., 2005]. The decrease in *ACAN* expression in chondrocytes from osteoarthritic articular cartilage did not correlate with increased methylation of the CpG islands in the *ACAN* promoter [Pöschl et al., 2005]. In other words, CpG methylation may not have a central role in the inhibition of aggrecan promoter activity during cartilage degradation [Pöschl et al., 2005]. Therefore, DNA methylation of *SOX9* could be a more relevant event eventually leading to cartilage degeneration. Nevertheless, the present study showed an apparently contradictory result regarding the roles of DNMTs on chondrogenesis of hBMSCs. DNMT3A and DNMT3B mRNA levels increased from day 3 onwards during the chondrogenic differentiation of hBMSCs, which would be after the peak period of SOX9 activity. The transient transfection performed herein during the initial stages of culture in fact enhanced the differentiation of hBMSCs without affecting the mRNA levels of SOX9. Possible explanations could be associated with distinct activities of DNMT3A or DNMT3B on suppressing the expression of yet unknown genes that inhibit the chondrogenic differentiation of MSCs. Alternatively, as shown in the present study, DNMT3A and DNMT3B could be inhibiting the transcription of stem-

ness-related genes, and promote a stronger differentiation of hBMSCs.

In conclusion, regulation of DNMT3A and DNMT3B activity by overexpression vectors or demethylating agent, 5-Aza, allowed discoveries on the function of these methyltransferases during chondrogenic differentiation of hBMSCs and could be useful tools for control of stemness and differentiation of stem cells for potential application in tissue engineering and cartilage regeneration.

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Disclosure Statement

The authors declare no competing interests.

Author Contributions

Y.N., Y.Y., H.T.N., S.N., T.K., and K.I. performed the experiments and analyzed the data. E.S.H. and M.O. designed and performed the experiments, analyzed the data, and wrote the manuscript. T.O. and T.K. analyzed the data and supported the study financially.

Availability of Data

All data are available upon request to the corresponding author.

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