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Ablation of Dnmt3b in chondrocytes suppresses cell maturation during embryonic development

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Abstract

DNA methylation is a major mode of epigenetic regulation in the mammalian genome and is essential for embryonic development. The three catalytic DNA methyltransferases (Dnmts), Dnmt1, Dnmt3a, and Dnmt3b, catalyze the methylation of cytosine. Dnmt3b is highly expressed in chondrocytes and global knockout of *Dnmt3b* led to skeletal deformations and embryonic lethality, suggesting an essential role of *Dnmt3b* in endochondral bone formation. To further define the role of *Dnmt3b* in skeletal development, *Dnmt3b* was deleted in *Col2* positive chondrocyte lineage cells. Both axial and appendicular skeletal size were reduced and bone mineralization delayed in *Col2Cre⁺;Dnmt3b^{f/f} (Dnmt3b^{Col2})* mice at E14.5 and E18.5. While Alcian Blue Hematoxylin/Orange G (ABH/OG) staining showed normal chondrocyte columns in control growth plates, the length of hypertrophic chondrocyte zone and type X collagen expression were decreased in E18.5 growth plates from *Dnmt3b^{Col2}* mice. TUNEL and PCNA staining demonstrated that the delay in chondrocyte maturation observed in the *Dnmt3b^{Col2}* growth plates was not secondary to altered chondrocyte apoptosis or proliferation. Complementary *in vitro* experiments were performed on primary sternal chondrocytes isolated from control and *Dnmt3b^{Col2}* mice. Gene expression studies confirmed delayed terminal maturation as *Mmp13* and *Col10a1* expression was down-regulated in *Dnmt3b^{Col2}* chondrocytes. In addition, alkaline phosphatase (ALP) and Alizarin Red staining confirmed that *Dnmt3b* deletion in chondrocytes delays *in vitro* chondrocyte hypertrophic differentiation and matrix mineralization. Mechanistically, *Dnmt3b* gene deletion resulted in decreased BMP signaling through reduction of Smad1 phosphorylation. These findings show that epigenetic factor, Dnmt3b is necessary for normal chondrocyte hypertrophic maturation and limb development.

Keywords

DNA methyltransferase 3b; chondrocyte; endochondral ossification; embryogenesis

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Introduction

Epigenetics is the regulation of gene expression in the absence of any changes in DNA sequence ⁽¹⁾. Three main mechanisms are involved in epigenetic regulation: 1) post-transcriptional modification of histones which alters chromatin conformation, 2) non-coding RNAs (i.e. microRNAs, long non-coding RNAs) that act post-transcriptionally to regulate of mRNA expression, and 3) DNA methylation ^(2–6). In DNA methylation CpG dinucleotide sequences are targeted by complementary enzymes that either methylate cytosine in GC rich regions of DNA (DNA methyltransferases; Dnmts) or that demethylate methyl-cytosine nucleotides (Ten-eleven demethylcytosine dioxygenases; TETs). DNA methylation regulates gene expression during embryonic development as well as during postnatal tissue growth and maintenance ⁽⁷⁾. DNA methylation involves the addition of a methyl group (CH₃) from the methyl donor S-adenosyl methionine (SAM) to a cytosine that lies 5' of guanine (CpG sites) to form methylated cytosine (5mC). CpG islands methylation frequently occurs in promoter, enhancer and gene body regions across the genome, differentially regulating gene expression profile, e.g. repetitive elements silencing, X chromosome inactivation, genomic imprinting, as well as regulation of gene expression via inhibition of transcription initiation ⁽⁸⁾.

There are three well documented catalytically active DNA methyltransferase (Dnmt) enzymes in mammalian cells, Dnmt1, Dnmt3a and Dnmt3b. Dnmt1 is ubiquitously expressed and is involved in maintaining the methylation signature of cells undergoing proliferation. Dnmt1 is thus important for expansion of various cell populations during development and in adult life ⁽⁹⁾. In contrast, Dnmt3a and Dnmt3b predominantly act as *de novo* DNA methyltransferases to establish lineage committed cells with tissue specific gene expression patterns by creating new DNA methylation signatures during cell differentiation ^(10,11). Deletion of these enzymes in mice results in embryonic (*Dnmt1* and *Dnmt3b*) or post-natal (*Dnmt3a*) lethality, confirming an essential role during development ^(12,13).

Given its prominent role in regulating gene expression and maintaining tissue specificity, *de novo* DNA methylation has been intensively studied in the context of development and disease. In neural stem cells, Dnmt3a is critical for neurogenesis since Dnmt3a-null mice have impaired postnatal neurogenesis and die 4 weeks after birth ^(13,14). In contrast, Dnmt3b gene deletion in neurons promotes cell differentiation ⁽¹⁵⁾. Dnmt3b was shown to be highly expressed in the superficial zone of normal articular cartilage, but with loss of expression occurring in conditions leading to the development of OA ⁽¹⁶⁾. Moreover, mice with conditional gene deletion of Dnmt3b in articular cartilage developed spontaneous osteoarthritis, further indicating a role in cartilage tissues ⁽¹⁶⁾. In the hematopoietic stem cells (HSCs), deletion of Dnmt3a and double knock out of Dnmt3a and 3b leads to marked decline in differentiation capacity, however, deletion of Dnmt3b alone in HSCs showed minor effect on cell differentiation ⁽¹⁷⁾. Global knockout of Dnmt3b in mice results in mid-gestation lethality due to multiple development defects including skeletal malformations. In humans, mutation of Dnmt3b occur in immunodeficiency, centromeric region instability, and facial anomalies (ICF) syndrome ⁽¹³⁾. This rare autosomal recessive disorder is characterized by variable immunological defects, centromeric heterochromatin instability, and facial anomalies ⁽¹⁸⁾, suggesting an essential role of Dnmt3b in endochondral ossification process

(13,19,20). Recently, Dnmt3b expression was shown to increase during the endochondral phase of fracture repair, and deletion of Dnmt3b in chondrocytes resulted in impaired fracture healing (21).

Mammalian skeletal and facial development primarily occurs through an endochondral ossification process, with calvaria developing through intramembranous ossification (22–25). The major difference between endochondral and intramembranous ossification is the formation of a cartilage anlagen which is a cartilage template for bone formation. At the outset of a highly ordered and regulated endochondral process, undifferentiated mesenchyme cells condense and differentiate into chondrocytes and form a type II cartilage matrix surrounded by perichondrium. In the cartilage template a zone of proliferation is succeeded by hypertrophic chondrocyte differentiation, type X collagen expression, and calcification of the matrix at the center of the cartilage anlagen. The calcified cartilage stimulates vascular invasion and serves as a subsequently serves as a template for primary bone formation. Eventually the center of the anlagen becomes mature bone and growth plates migrate towards opposite ends of the long bone. Therefore, chondrocyte hypertrophy is the central event bridging between chondrocyte proliferation and bone formation during the long bone elongation process.

In these studies, we determine the importance of Dnmt3b during the cartilage phase of skeletal development in using mice with conditional deletion of Dnmt3b in chondrocytes. Our findings confirm that Dnmt3b is a critical epigenetic factor that regulates chondrocyte hypertrophy, potentially through modulation of the BMP signaling pathway during the endochondral ossification process.

Materials and methods

Animals

Col2a1-Cre mice were obtained from Jackson Laboratory (26,27). *Dnmt3b^{fl/fl}* mice, generated by Dr. En Li, were obtained from Mutant Mouse Regional Resource Centers (MMRRC) (28). To generate *Col2-Cre;Dnmt3b^{fl/fl}* (*Dnmt3b^{Col2}*) mice, *Dnmt3b^{fl/fl}* mice were crossed with *Col2a1-Cre* transgenic mice. The Cre-negative littermates were used as controls. All the procedures were approved by Washington University Animal Studies Committee.

Whole skeleton preparation

Whole mount Alizarin red-Alcian blue staining was used to determine the embryonic skeleton composition in embryonic day 14.5 (E14.5) and E18.5 following protocols described by McLeod (29). Briefly, After (29). Briefly, after fixation in 95% ethanol for 24h, s, all the samples were placed in 95% ethanol-Alcian blue and Alizarin red solution (24 and 48h respectively) for cartilage and bone staining. Samples were washed with 95% ethanol (8h) and incubated and in 1% KOH overnight at 4 °C. Samples were then cleared with 20, 50, and 80% glycerol in 1% KOH for 12h respectively and stored in 100% glycerol. Analyses of the skeletal phenotype between genotypes were performed with Encoded stereo microscopes (Leica M125, Leica Microsystems, US). Quantitative measurements of body

size-crown-rump length were performed by Image J 1.46r (Wayne Rasband, National Institutes of Health, US).

Immuno- and histological analyses

Hind limb samples harvested from E18.5 embryos were fixed in 10% formalin overnight. Following decalcification and processing, specimens were sectioned at a thickness of 5µm. Alcian Blue Hematoxylin/Orange G (ABH/OG) staining was performed to analyze skeletal architecture and cartilaginous compartment organization. Stained sections were scanned by Hamamatsu NanoZoomer 2.0-HT System and captured by NDP.view2 Viewing software (U12388-01) for image viewing.

The expression pattern of Dnmt3b, Col10a1, phospho-Smad1 and phospho-Smad3 was examined by immunohistochemistry on E18.5 tibia sections. Briefly, after de-paraffinization and rehydration, antigen retrieval was performed by heat and pressure for Dnmt3b or pepsin digestion for Col10a1. Dnmt3b (LSBio, #LS-B1191, 1:1000), Col10a1 (Quartett, #2031501005, 1:200), phospho-Smad1 (Cell Signaling, #9516, 1:800) and phospho-Smad3 (Abcam, #ab52903, 1:200) antibodies were used respectively for overnight incubation. For Dnmt3b, NovaRED Peroxidase (HRP) Substrate Kit (Vector Laboratories, #SK-4800) was used for colorimetric development and counterstaining was performed using hematoxylin stain (Vector Laboratories, #H-3404). Images were captured with a light microscope (Zeiss Axio Scope A1, Carl Zeiss Co, Ltd, Jena, Germany). Dnmt3b-positive cells were quantified from at least 3 animals of each genotype by Image J 1.46r. For Col10a1, phospho-Smad1 and phospho-Smad3, tyramide Signal Amplification Kit (Invitrogen, #T20912) was used to generate fluorescent signals and the cell nuclei were stained with DAPI (Vector Laboratories, #H-1200). The fluorescence was detected by Microscope Axio Imager.D2 (Carl Zeiss Co, Ltd, Jena, Germany). The length of the Col10a1 positive cartilage compartment was measured by Image J 1.46r.

PCNA and TUNEL staining

PCNA staining was performed to examine cell proliferation from histology sections. After de-paraffinization and rehydration, heat-induced antigen retrieval was performed with citric acid buffer (pH 6.0). Rabbit anti-mouse antibody against PCNA (CST, #13110, 1:500) was incubated overnight at 4°C. Tyramide Signal Amplification Kit (Invitrogen, #T20912) was used to generate fluorescent signals and the cell nuclei were stained with DAPI. Fluorescence was detected using a Microscope Axio Imager.D2. PCNA positive cells were quantified by Image J 1.46r and the percentage of PCNA-positive cell was quantified from at least 3 animals of each genotype.

TUNEL assay was performed on paraffin sections with In Situ Cell Death Detection Kit (Roche, #11684795910) according to the manufacturer's instructions. Briefly, after de-paraffinization and rehydration, cells were permeabilized with 10µl/ml Proteinase K in 10mM Tris/HCL (PH7.4-8) at room temperature for 20min. TUNEL reaction mixture was then added to each sample and incubated for 60min at 37°C in a humidified chamber in the dark. The cell nuclei were stained with DAPI (Vector Laboratories, #H-1200) and the fluorescence was detected by Microscope Axio Imager.D2.

Primary murine costal chondrocyte isolation and culture

Costal chondrocytes were isolated from sternae and ribs of P2–P5 WT or *Dnmt3b^{Col2}* neonatal pups as previously described⁽³⁰⁾. Briefly, sternae and ribs were dissected and pre-digested with 2 mg/ml pronase and 3 mg/ml collagenase D at 37 °C for 1h, respectively. This was followed with the second 4–6 h collagenase D digestion. Primary chondrocytes were then harvested and filtered by 40µm cell strainer. The cell suspensions were seeded at a density of 250,000 cells/well in 24-well tissue culture plates in HG-DMEM medium supplemented with 10% FBS. Chondrocytes were subsequently placed in maturation medium, including 50µg/mL L-Ascorbic acid (Sigma, #A4544) and 10mM β-Glycerophosphate disodium salt hydrate (Sigma, #G9422) in HG-DMEM medium⁽³¹⁾. The chondrocyte maturation medium was changed every 3 days and the cells were cultured for 5, 14, and 21 days.

Real-time qPCR and Western blot

RNA was isolated from chondrocyte cultures using RNeasy Mini Kit (QIAGEN, #74104). cDNA synthesis and real-time qPCR were performed according to the protocols of the manufacturer. Primer sequences are shown in Table 1. Western blot was performed to examine the protein level of phospho-Smad1, Smad1, phospho-Smad3, Smad3 and β-actin from control and *Dnmt3b^{Col2}* chondrocytes. The following primary antibodies were used: phospho-Smad1 (Cell Signaling, #9516, 1:500), Smad1 (Cell Signaling, #6944, 1:1000), phospho-Smad3 (Abcam plc., #ab52903, 1:2000), Smad3 (Abcam plc., #ab40854, 1:2000) and β-actin (Sigma, #2228, 1:2000). Quantifications of the protein expressions (relative intensity) were measured by Image J 1.46r software.

Alkaline phosphatase (ALP) and Alizarin Red staining

Chondrocyte maturation and mineral deposition were examined by ALP and Alizarin Red staining respectively. Briefly, following fixation with 10% formalin, the cell cultures were stained by NBT/BCIP substrate solution to detect ALP activity or alizarin red solution to detect mineral deposition. After washing each well three times with H₂O, images were captured by scanner (EPSON, Perfection 1660).

Statistical Analysis

All quantitative data were presented as mean ± standard deviation with a minimum of three independent experiments. Statistical significance was determined by two-tailed Student's t test. *p* < 0.05 was considered statistically significant.

Results

Ablation of *Dnmt3b* in chondrocytes reduces embryonic skeletal growth

In order to investigate the role of *Dnmt3b* during the endochondral ossification process, *Col2Cre; Dnmt3b^{f/f}* mice (*Dnmt3b^{Col2}*) were generated to specifically delete *Dnmt3b* in chondrogenic lineage cells⁽³²⁾, which compensates for the embryonic lethality of global knockout of *Dnmt3b* in mice. The deletion efficiency was confirmed by immunohistochemistry (IHC) in the hind limb of E18.5 embryos. IHC showed that *Dnmt3b*

was ubiquitously expressed in E18.5 WT chondrocytes, including both proliferating and hypertrophic chondrocytes, whereas the expression of *Dnmt3b* was markedly reduced in *Dnmt3b^{Col2}* chondrocytes (Fig. 1A and B).

The *Dnmt3b^{Col2}* mice were born at the expected Mendelian ratio with no gross abnormality. The whole mount skeletal staining of the embryos at E14.5 and E18.5 indicated that the skeletal elements mineralized during development in both WT and *Dnmt3b^{Col2}* mice, however, the whole skeletal size was reduced in the mutant embryos compared to their littermate controls (Fig. 1C). Quantitative measurement confirmed that body size-crown-rump length was significantly decreased in *Dnmt3b^{Col2}* embryos at E14.5 and E18.5 (Fig. 1D). Further evaluation of the limb skeletal elements demonstrated that the total length (TL) of the humerus, femur, radius, or tibia was shortened by 10% to 25% in *Dnmt3b^{Col2}* embryos compared to the littermates (Fig. 2, Fig. S1). Similarly, the width of the diaphysis of the humerus and tibia (BW; measured at the mid-point of the bone shaft) was reduced by 10% in *Dnmt3b^{Col2}* embryos (Fig. 2, Fig. S1). Interestingly, while the cartilage length (CL) of each bone was not significantly different between WT and mutant embryos, the bone shaft length (BL) of each long bone was decreased in *Dnmt3b^{Col2}* embryos compared to WT embryos (Fig. 2, Fig. S1).

Consistent with the whole skeletal observation, histological analyses of E18.5 tibia by ABH/OG staining confirmed the shortening of bone shaft in *Dnmt3b^{Col2}* embryos (Fig. 2B and C). The epiphyseal cartilage contains resting chondrocyte in the round zone and proliferative chondrocytes in the columnar zone⁽³³⁾. Further examination in cartilage tissue revealed no difference in the morphology of the chondrocytes or the organization in either round or columnar zone between the genotypes (Fig. 2B). The cell density in E18.5 *Dnmt3b^{Col2}* embryos was similar between in the round zone and in the columnar zone of mutant and WT embryos, suggesting that chondrocyte hypertrophy and maturation is impaired by ablation of *Dnmt3b*, but not chondrocyte proliferation (Fig. S2). Altogether, *Dnmt3b* is essential for the embryonic bone growth, but does not affect the morphology and proliferation of cartilage tissue.

***Dnmt3b* stimulates chondrocyte hypertrophic maturation**

Since chondrocyte hypertrophic maturation is required for longitudinal growth of long bones during embryogenesis, we isolated primary murine costal chondrocyte from *Dnmt3b^{Col2}* mice and control mice and cultured in chondrocyte maturation medium for up to 21 days to stimulate cell maturation. RT-PCR data confirmed that *Dnmt3b* expression was reduced by 80% after 5 days culture, whereas, the expression of *Dnmt1* and *Dnmt3a* was not affected in *Dnmt3b^{Col2}* chondrocytes (Fig. 3A). The chondrocyte maturation makers, *Mmp13* and *Col10a1*, were suppressed in *Dnmt3b^{Col2}* chondrocytes compared to control chondrocytes (Fig. 3A). Consistent with the gene expression profile, ALP staining and Alizarin Red staining also showed a delay in chondrocyte maturation in chondrocytes with deletion of *Dnmt3b*, with reduced ALP activity and mineral deposition in *Dnmt3b^{Col2}* chondrocyte cultures compared to control cultures after 21 days in chondrocyte differentiation medium (Fig. 3B). Furthermore, immunofluorescence for COL10A1 in E18.5 limb revealed that Type X Collagen was highly expressed in the hypertrophic chondrocytes of E18.5 mice,

whereas the expression of COL10A1 was significantly reduced in *Dnmt3b^{Col2}* embryos (Fig. 3C). Quantification of the length of COL10A1-positive cells demonstrated a 35% reduction in the length of the COL10A1 zone in E18.5 mutant embryos compared to littermate controls (Fig. 3D). Chondrocyte proliferation and apoptosis were examined in *Dnmt3b^{Col2}* chondrocytes and no difference was observed in cell proliferation in either chondrocytes in the reserve zone or chondrocytes in the proliferating zone of the proximal tibia in *Dnmt3b^{Col2}* and littermate control embryos at E18.5 days (Fig. 4A and B). Similarly, no difference in TUNEL staining was observed (Fig. 4C). Altogether, loss of Dnmt3b suppressed chondrocyte maturation and led to the defect of long bone longitudinal growth.

Dnmt3b regulates chondrocyte maturation through BMP pathway

TGF- β /BMP pathway is well documented in regulating chondrocyte maturation and homeostasis *in vitro* and *in vivo*, therefore, protein was isolated from control and *Dnmt3b^{Col2}* chondrocytes to assess the TGF- β /BMP signaling pathway. Western blot was performed to determine the expressions of Smad1, Smad3, phospho-Smad1 and phospho-Smad3. Western blot revealed no difference in the TGF- β pathway but showed down-regulation of the BMP pathway in *Dnmt3b^{Col2}* chondrocytes as indicated by decreased expression of phospho-Smad1 (Fig. 5A). Quantifications of the protein expressions confirmed that the ratio of phospho-Smad1/Smad1 was reduced, while the ratio of phospho-Smad3/Smad3 maintained unchanged in *Dnmt3b^{Col2}* chondrocytes compared with WT cells (Fig. 5B). Immunofluorescence for phospho-Smad1 and phospho-Smad3 in E18.5 limb confirmed that the expression of phospho-Smad1 was significantly decreased in the hypertrophic chondrocytes of E18.5 mice (Fig. 5C), whereas the expression of phospho-Smad3 was not altered in *Dnmt3b^{Col2}* embryos (Fig. S3), indicating Dnmt3b regulates the chondrocyte maturation through BMP pathway during embryonic development.

Discussion

The DNA methyltransferases are critical in development and normal tissue function, and are involved in aging, cancer, and in disease pathogenesis. Here we defined the biological functions of the *de novo* Dnmt3b in the regulation of embryonic endochondral bone development. Previous studies from human chondrocytes reported the expression of Dnmts in human chondrocytes⁽³⁴⁾, however the role of Dnmts in chondrocytes during embryonic skeletal development has not yet been examined. In this study, we examined the function of Dnmt3b in Col2 positive chondrocyte lineage and found that ablation of Dnmt3b in chondrocytes suppressed chondrocyte hypertrophic differentiation and skeletal growth at E14.5 and E18.5, at least partially through down-regulation of BMP signaling pathway during endochondral bone formation process.

In mammals, three conserved catalytically active enzymes, Dnmt1, Dnmt3a and Dnmt3b, are responsible for DNA methylation and maintenance of methylation during cell proliferation and are indispensable for normal development^(13,35). During DNA replication, Dnmt1 mediates epigenetic inheritance in dividing cells through the duplication of methylation on the new DNA strand at hemimethylated CpG sites. Thus loss of Dnmt1 leads to whole genome DNA methylation collapse⁽³⁶⁾. On the other hand, unmethylated CpGs are

specifically targeted by Dnmt3a and Dnmt3b during *de novo* methylation which plays important role to maintain cell proliferation, differentiation as well as tissue homeostasis and specificity (37–39). Recently, high-throughput screenings have characterized the role and distribution of DNA methylation during embryonic development. It has been documented that the methylation primarily occurred in the gene locus which regulates cellular identity and genomic stability during early embryogenesis (40). For developmental transitions, DNA methylation exhibits a repression function on the germline expression program during implantation at the transition from the blastocyst to the post-implantation epiblast and is also targeted to promoters of lineage-specific genes in the blastocyst such as hematopoietic genes with demethylation during terminal differentiation (41). It is worth to note that both of these dynamics on DNA methylation during early embryogenesis is mediated by Dnmt3a and Dnmt3b (41) and in general, Dnmt3b has high activity *in vitro* (42).

Several studies from mouse embryogenesis indicate an overlapping role Dnmt3a and Dnmt3b in regulating of growth, morphogenesis and gene silencing and yet, Dnmt3a and Dnmt3b also have distinct functions (13,43,44). During early embryonic development, the phenotype that abnormal morphology and lethality of Dnmt3a^{-/-};Dnmt3b^{-/-} double homozygous embryos was apparently more severe than that of Dnmt3a^{-/-} or Dnmt3b^{-/-} single mutant embryos, demonstrating the presence of overlapping function between Dnmt3a and Dnmt3b (13). The irreplaceable role of Dnmt3a is specifically exhibited in the methylation of imprinted genes in germ cells (45) and the Xist promoter region on the X chromosome (39). Moreover, Dnmt3a expression in neural stem cells is essential for neurogenesis. In neurogenesis, Dnmt3a targets non-proximal promoter methylation sites to maintain an active chromatin state of genes critical for neural development (46). During osteoclastogenesis, osteoclast precursors continuously express mRNA for Dnmt3a, which plays an important role in the regulation of cellular metabolism and differentiation in bone disorders (47). On the other hand, for the minor satellite repeats during the embryonic development, its methylation is primarily in the control of Dnmt3b without contribution from Dnmt3a, indicating Dnmt3b specifically required for methylation of centromeric minor satellite repeats in development (13). Furthermore, in mammalian embryonic stem cells (ESCs), Dnmt3b could also interact with histone modification enzymes to regulate the accessibility of gene transcription machinery to specific DNA loci during embryo development. (48). Despite the redundancy of Dnmt3a and Dnmt3b in cells and tissues, Dnmt3a still needs to be further explored to fully delineate the role of *de novo* DNA methylation during skeletal development process.

As the most extensively studied epigenetic marks, DNA methylation is considered to be essential to transcriptional gene silencing during mammalian development (49). In mammalian genomes, DNA methylation ordinarily emerges at the special regions of promoters and enhancers of inactive genes, or at repetitive elements and within transcribed gene bodies (50–52). In particular, DNA methylation at the promoter region has been related to gene activity through direct influence on gene expression patterns and cellular phenotype (53). Although DNA methylation are highly studied epigenetic modifications, the exact role of DNA methylation in regulating genome function and how this differs from gene to gene and in genomic context need to be uncovered (49). It still remains to be fully understood how specific DNA methylation patterns are precisely generated, maintained, read, or erased along

the genome. In this regard, Genome-wide methylation screen is necessary to identify the methylation patterns of TGF/BMP pathway related molecules, including Smad1, 3, 4 etc. in the promoter, enhancer and gene body region. Moreover, DNA methylation is a dynamic process and Ten-eleven Translocation Enzymes (Tets) are involved in the DNA demethylation, thus, Tets may contribute to the chondrocyte maturation and skeletal development as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Ablation of Dnmt3b in chondrocytes inhibited embryonic skeletal growth

(A) Immunohistochemical analyses of Dnmt3b in hind limbs sections from WT and *Dnmt3b^{Col2}* embryos at E18.5. Data are representative of three independent experiments. Scale bar, 50µm. (B) Quantifications of Dnmt3b-positive cells were performed on hind limbs sections from WT and *Dnmt3b^{Col2}* embryos at E18.5. Data are expressed as means $\pm$ SD of three independent experiments. *, $p < 0.05$ compared with WT group by two-tailed Student's *t* test. (C) The whole mount skeletal staining was performed on WT and *Dnmt3b^{Col2}* embryos at E14.5 and E18.5. Data are representative of three independent experiments. (D) Quantifications of body size-crown-rump length (CRL) were performed on WT and *Dnmt3b^{Col2}* embryos at E14.5 and E18.5. Data are expressed as means $\pm$ SD of three independent experiments. *, $p < 0.05$ compared with WT group by two-tailed Student's *t* test.

Fig. 2. Ablation of Dnmt3b in chondrocytes results in reduced limb length

(A) Alizarin red-Alcian blue staining was performed on the fore- and hind limbs of *Dnmt3b^{Col2}* and littermate control embryos at E14.5 and E18.5. Data are representative of three independent experiments. (B) Hematoxylin/Orange G (ABH/OG) staining was performed on the hind limbs sections of *Dnmt3b^{Col2}* and littermate control embryos at E18.5. Reserve zone chondrocytes (red box) and in proliferating columnar zone of chondrocytes (green box) in panel B1-B2 are shown at high magnification in panels B3-B4 and in panels B5-B6, respectively. Data are representative of five independent experiments.

Scale bar, 0.5mm. (C) Measurements of the length of the region of bone formation in the mid-shaft, cartilage compartment and hypertrophic zone were performed on the hind limbs sections of *Dnmt3b^{Col2}* and littermate control embryos at E18.5. All results were normalized to respective measurements in the littermate control group at E18.5, which were set at 1.0. Data are expressed as means $\pm$ SD of five independent experiments. *, $p < 0.05$ compared with WT group by two-tailed Student's *t* test.

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Fig. 3. Loss of Dnmt3b in chondrocytes suppressed hypertrophic maturation

(A) Real-time qPCR analyses were performed to determine the relative expression of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Col10a1* and *Mmp13* in primary chondrocytes isolated primary murine costal chondrocyte from P2–P5 *Dnmt3b^{Col2}* mice and littermate control mice, which were cultured in chondrocyte maturation medium up to 21 days. The mRNA levels were normalized to that of the gene coding β -actin and then were normalized to the littermate control group. Data are means $\pm$ SD of three independent experiments. *, $p < 0.05$ compared with WT group by two-tailed Student's *t* test. (B) Alkaline phosphatase (ALP) and Alizarin

Red staining of *Dnmt3b^{Col2}* and littermate control chondrocytes cultured in chondrocyte maturation medium for up to 21 days. Data are representative of three independent experiments. (C) The immunofluorescence for COL10A1 was performed on the hind limbs sections of *Dnmt3b^{Col2}* and littermate control embryos at E18.5. Data are representative of five independent experiments. The growth plate (red box) in panels A1-A2 are shown at high magnification in panels A3-A4, respectively. Scale bar, 100µm. (D) Quantifications of the length of COL10A1-positive cells (between yellow arrow) were performed on the hind limbs sections *Dnmt3b^{Col2}* and littermate control embryos at E18.5. Data are expressed as means ± SD of five independent experiments. *, $p < 0.05$ compared with WT group by two-tailed Student's *t* test.

Fig. 4. Loss of Dnmt3b does not alter chondrocyte proliferation or apoptosis
 (A) PCNA staining was performed on the hind limbs sections of *Dnmt3b^{Col2}* and littermate control embryos at E18.5. Reserve zone chondrocytes (yellow box) and proliferating columnar zone chondrocytes (green box) in panel A1-A2 are shown at high magnification in panels A3-A4 and in panels A5-A6, respectively. Data are representative of five independent experiments. Scale bar, 100µm. (B) Quantifications of the percent of PCNA-positive cells were performed in the reserve zone and columnar proliferating zone of the proximal tibia sections of *Dnmt3b^{Col2}* and littermate control embryos at E18.5. Data are expressed as

means $\pm$ SD of five independent experiments. *, $p < 0.05$ compared with the littermate control group by two-tailed Student's t test. (C) TUNEL staining was performed on the hind limbs sections of *Dnmt3b*^{Col2} and littermate control embryos at E18.5. Reserve zone chondrocytes (yellow box) and columnar zone chondrocytes (green box) in panel A1-A2 are shown at high magnification in panels A3-A4 and A5-A6, respectively. Data are representative of five independent experiments. Scale bar, 100 μ m.

Fig. 5. Dnmt3b regulates chondrocyte maturation through BMP pathway during embryonic development

(A) Western blot was performed to examine the protein level of phospho-Smad1, Smad1, phospho-Smad3, Smad3 and β-actin from *Dnmt3b^{Col2}* and littermate control chondrocytes cultured in chondrocyte maturation medium for up to 21 days. Data are representative of three independent experiments. (B) Quantifications of the protein expressions were obtained using Image J 1.46r software. Data are expressed as means ± SD of three independent experiments. *, $p < 0.05$ compared with WT group by two-tailed Student's *t* test. (C) The immunofluorescence for phospho-Smad1 was performed on the hind limbs sections of

Dnmt3b^{Col2} and littermate control embryos at E18.5. Data are representative of three independent experiments. The area (red box) in panels A5-A6 are shown at high magnification in panels A7-A8, respectively. Scale bar, 50µm. (D) Quantifications of pSmad1-positive cells were performed on hind limbs sections from WT and *Dnmt3b*^{Col2} embryos at E18.5. Data are expressed as means ± SD of three independent experiments. *, p < 0.05 compared with WT group by two-tailed Student's *t* test.

Table 1

Primer sequences for real-time qPCR

Genes	Sequences
<i>Dnmt1</i>	5'- CCT GCC AGG GCT TCA GTG GC-3' 5'- CAG GCA GCG CAG TGT GAG CT-3'
<i>Dnmt3a</i>	5'- GCC GAA TTG TGT CTT GGT GGA TGA CA-3' 5'- CCT GGT GGA ATG CAC TGC AGA AGG A-3'
<i>Dnmt3b</i>	5'- AAT ACC CAA CTC CTT GAG CAC-3' 5'- TCT TCA CTA CTG ATC CTG ACC T-3'
<i>Col10a1</i>	5'-ATG CCT TGT TCT CCT CTT ACT G-3' 5'-TGC TGA ACG GTA CCA AAC G-3'
<i>Mmp13</i>	5'-AGA CTG GTA ATG GCA TCA AGG-3' 5'-GCC ATT TCA TGC TTC CTG ATG-3'
<i>Actin</i>	5'- AGA TGT GGA TCA GCA AGC AG-3' 5'-GCG CAA GTT AGG TTT TGT CA-3'