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Histone Demethylase Utx Regulates Differentiation and Mineralization in Osteoblasts

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ABSTRACT

Alteration of methylation status of lysine 27 on histone H3 (H3K27) associates with dramatic changes in gene expression in response to various differentiation signals. Demethylation of H3K27 is controlled by specific histone demethylases including ubiquitously transcribed tetratricopeptide repeat X chromosome (Utx). However, the role of Utx in osteoblast differentiation remains unknown. In this study, we examined whether Utx should be involved in osteoblast differentiation. Expression of Utx increased during osteoblast differentiation in MC3T3-E1 cells and primary osteoblasts. GSK-J1, a potent inhibitor of H3K27 demethylase, increased the levels of trimethylated H3K27 (H3K27me3) and decreased the expressions of Runx2 and Osterix and ALP activity in MC3T3-E1 cells. Stable knockdown of Utx by shRNA attenuated osteoblast differentiation and decreased ALP activity, calcium content, and bone-related gene expressions. Silencing of Utx increased the level of H3K27me3 on the promoter regions of Runx2 and Osterix and decreased the promoter activities of Runx2 and Osterix. Taken together, our present results propose that Utx plays important roles in osteoblast differentiation by controlling the expressions of Runx2 and Osterix. J. Cell. Biochem. 116: 2628–2636, 2015.

KEY WORDS: Utx; H3K27ME3; OSTEOBLAST DIFFERENTIATION; HISTONE DEMETHYLATION; RUNX2; OSTERIX

Bone formation is tightly regulated processes that are characterized by a sequence of events starting by the commitment of osteoprogenitor cells. Osteoprogenitor cells in turn differentiate into pre-osteoblasts and then mature osteoblasts. Osteoblasts synthesize bone matrix and maintain a certain levels of bone mass and calcium homeostasis. The differentiation of osteoblasts from their precursors is regulated by specific transcriptional factors Runx2 and Osterix through controlling the expression of osteoblast marker genes including osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) [Nakashima et al., 2002; Neve et al., 2013; Okamura et al., 2013]. In fact, embryos of Runx2- or Osterix-null mouse do not express these osteoblast differentiation markers [Tai et al., 2004; Marie, 2008; Sinha and Zhou, 2013].

Posttranslational histone modifications including methylation are closely linked to regulation of eukaryotic gene expression. Histone methylation mainly occurs on lysine residues. Lysine residues of histone can be mono-, di-, or tri-methylated and the degree of methylation influences which proteins can bind to chromatin and modify the chromatin structure [Kouzarides, 2007; Campos and Reinberg, 2009]. Gene-wide mapping of chromatin state in differentiated cells has revealed that methylation status of histone H3 is a potential mark in the promoter regions of related-genes [Mikkelsen et al., 2007; Zhao et al., 2007]. For instance, trimethylation on histone H3 lysine 27 (H3K27me3) is associated with transcriptional repression, whereas trimethylation on H3 lysine 4 (H3K4me3) is found at sites of active transcription [Schübeler et al., 2004; Barski et al., 2007; Zhou et al., 2011]. These marks seem to maintain pluripotency of embryonic stem cells and prime those genes for activation in response to some differentiation signals [Mikkelsen et al., 2007; Zhao et al., 2007; Wei et al., 2009].

The removal process of H3K27 methylation is thought to play a pivotal role in lineage determination of many types of cells. The discovery of the demethylases of H3K27me3, ubiquitously

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transcribed tetratricopeptide repeat X chromosome (Utx) and Jumonji domain containing three (Jmjd3), has increased our understanding how developmental process is regulated by histone methylation. Utx contains Jumonji C-domain required for histone demethylase activity, which shares 84% of sequence similarity to that of Jmjd3 [Agger et al., 2007; Hong et al., 2007]. Utx is ubiquitously expressed and Utx-null mice are embryonic lethal with defects in cardiac development and neural tube closure [Lee et al., 2012; Welstead et al., 2012]. Utx regulates stem cell migration and hematopoiesis [Thieme et al., 2013], and is a key factor for embryonic development [Morales Torres et al., 2013]. We have recently reported that Jmjd3 regulates osteoblast differentiation and bone formation through the histone modification on the promoter regions of Runx2 and Osterix [Yang et al., 2013]. However, whether Utx is involved in osteoblast differentiation is still unknown.

The purpose of this study was to examine Utx expression and its function in osteoblast differentiation. To better understand the role of Utx during osteoblast differentiation, we used an inhibitor of Utx and a short hairpin RNA (shRNA) approach and examined the consequences of modulating Utx activity and expression on osteoblast differentiation. We demonstrate that Utx has important roles in osteoblast differentiation by regulating the transcription factors Runx2 and Osterix.

MATERIALS AND METHODS

MATERIALS

Alpha-modified Eagle's minimal essential medium (α-MEM) was purchased from Invitrogen (Carlsbad, CA). Plastic dishes were from IWAKI (Chiba, Japan) and fetal bovine serum (FBS) from JRH Biosciences (Lenexa, KS). Antibody against Utx was purchased from Abcam (Cambridge, UK). Antibodies against for H3K27me3, H3K4me3, H3K9me3, H3K36me3, and H3 were obtained from Takara (Shiga, Japan). Anti-β-actin antibody, ascorbic acid (ASA), β-glycerophosphate (β-GP), Fast Red TR, and naphthol AS-MX phosphate were purchased from Sigma–Aldrich (St. Louis, MO). The other materials used were of the highest grade commercially available. MC3T3-E1 cells were obtained from Riken Cell Bank (Tsukuba, Japan).

CELL CULTURE

Primary osteoblasts were prepared from Balb/c mouse calvaria as described previously [Miyai et al., 2009]. All mice studied were reared in our specific pathogen-free mouse colony and given food and water ad libitum. Experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of Tokushima University, Tokushima, Japan (toku–dobutsu 10051). MC3T3-E1 cells and primary osteoblasts were cultured in α-MEM supplemented with 10% FBS at 37°C under a humidified atmosphere of 5% CO_2. For the induction of osteoblast differentiation, the growth medium was supplemented with 50 μM ASA and 10 mM β-GP (osteoblast differentiation medium). For the experiments using inhibitor, cells were pretreated with GSK-J1 (Tocris Bioscience, Bristol, UK) for 2 h and then cultured in the osteoblast differentiation medium for the indicated periods.

RNA PREPARATION AND REAL-TIME PCR ANALYSIS

Cultured cells were homogenized in Trizol reagent (Invitrogen), and total RNA was extracted according to the manufacture's protocol. Reverse transcription was carried out with Reverse Transcription Kit (Takara). Real-time PCR of each gene was performed in triplicate for at least three independent experiments with a 7300 Real-time PCR system (Applied Biosystems, Carlsbad, CA) using SYBR Premix Ex Taq™ (Takara). The sequences of the primers are as follows:

Gapdh:
forward, 5′-TGTGTCCGTCGTGGATCTGA-3′
reverse, 5′-TTGCTGTTGAAGTCGCAGGAG-3′
Utx:
forward, 5′-TATTGGCCCAGGTGACTGTGAA-3′
reverse, 5′-CAGATCTCCAGGTCGCTGAATAAAC-3′
Runx2:
forward, 5′-CATTTGCACTGGGTCACACGTA-3′
reverse, 5′-GAATCTGGCCATGTTTGTGCTC-3′
Osterix:
forward, 5′-CTTCCCAATCCTATTTGCCGTTT-3′
reverse, 5′-CGGCCAGGTTACTAACACCAATCT-3′
OCN:
forward, 5′-CCGGGAGCAGTGTGAGCTTA-3′
reverse, 5′-AGGCGGTCT TCAAGCCATACT-3′
OPN:
forward, 5′-TACGACCATGAGATTGGCAGTGA-3′
reverse, 5′-TATAGGATCTGGGTGCAGGCTGTAA-3′
BSP:
forward, 5′-GAGCCTCGTGGCGACACTTA-3′
reverse, 5′-AATTCTGACCCTCGTAGCCTTCATA-3′

SDS-PAGE AND WESTERN BLOT ANALYSIS

Cultured cells were washed twice with phosphate-buffered saline (PBS) and then scraped into lysate buffer (1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM EGTA). The protein concentration was determined by using Protein Assay Reagent (Bio–Rad, Hercules, CA) and diluted to a concentration of 1 mg/ml with lysate buffer. Twelve micrograms of each sample and pre-stained molecular weight markers (Bio–Rad) were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Medford, MA). The membranes were incubated for 2 h at ambient temperature in a blocking solution consisting of 5% non-fat skim milk in PBS containing 0.05% Tween-20 (PBS-Tween), washed briefly in PBS-Tween, and then incubated overnight at 4°C in 5% non-fat skim milk in PBS-Tween containing specific antibodies (diluted at 1:1,000). After the membranes had been washed 4 times within 30 min in PBS-Tween, they were incubated at ambient temperature for 2 h in PBS-Tween containing horseradish peroxidase-conjugated secondary antibodies (diluted at 1:5,000). The membranes were then washed again as described above, and the proteins recognized by the antibodies were visualized with an ECL detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions.

SHORT HAIRPIN RNA (shRNA) TRANSFECTION

For establishment of stable Utx knockdown cells, Utx shRNA lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA) were

infected into the cells according to the manufacturer's directions and selected the stable clones via puromycin (5 μg/ml) treatment (shUtx). MISSION® Non-Target shRNA lentiviral transduction particles (Sigma–Aldrich) were used as a negative control (shCont). The target sites were as follows:

shUtx: UTX shRNA Lentiviral Particles (sc-76881-V), Santa Cruz Biotechnology;

shCont:

5′-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTC-TTCATCTTGTTGTTTTT-3′

REPORTER CONSTRUCTS AND LUCIFERASE ASSAY

To clone the 5′ upstream region of Runx2, −625/+1 of the Runx2 gene was amplified by PCR from DNA extracted from MC3T3-E1 cells. PCR product was digested with XhoI and Hind III and inserted into the pGL3 basic luciferase reporter vector (Promega, Madison, WI). Osterix promoter −786/+91 reporter was kindly provided from Drs. Tohmonda and Horiuchi (Keio University, Japan). For luciferase assays, 70–80% confluent cells in 24-well dishes were transfected with 0.5 μg of promoter reporter vector using Lipofectamine LTX™ reagent (Invitrogen) according to the manufacturer's directions. The cells were also co-transfected with 0.05 μg of pTK-*Renilla* (Promega) to normalize for transfection efficiency. pGL3 basic vector (Promega) was used for empty vector as control. After 24 h post-transfection, cell lysates were prepared using Dual-Glo® Luciferase Assay System (Promega) and assessed for the luciferase activity.

ALKALINE PHOSPHATASE (ALP) STAINING

MC3T3-E1 cells cultured for seven days in the osteoblast differentiation medium were fixed in 3.7% formaldehyde for 10 min and stored at 4°C in 100 mM cacodylic acid buffer (pH 7.4). The cells were then incubated at 37°C with freshly prepared alkaline phosphatase substrate solution (100 mM Tris–maleate buffer (pH 8.4), 2.8% *N*, *N*-dimethyl formamide (v/v), 1 mg/ml Fast Red TR, and 0.5 mg/ml naphthol AS-MX phosphate). The reaction was terminated after 30 min by removal of the substrate solution and washing with 100 mM cacodylic acid buffer.

ALP ACTIVITY ASSAY

MC3T3-E1 cells cultured for seven days in the osteoblast differentiation medium were scraped into ice-cold 50 mM Tris–HCl buffer (pH 7.4), sonicated for 20 sec using a sonifier-cell disruptor (Model UR-20P; TOMY, Tokyo, Japan), and centrifuged at 10,000 × g for 20 min at 4°C. The ALP activity in the supernatant was then determined using *p*-nitrophenyl phosphate as a substrate according to the manufacturer's instructions. The ALP activity was normalized to the protein content as measured by Protein Assay Reagent (Bio–Rad).

MINERAL DEPOSITION AND QUANTIFICATION

For Von Kossa staining, MC3T3-E1 cells cultured for 14 days in the osteoblast differentiation medium were fixed in 3.7% formaldehyde for 10 min, washed in cacodylic acid buffer pH 7.4, incubated in saturated lithium carbonate, and subsequently incubated in 3% $AgNO_3$ (w/v) for 30 sec under ultraviolet light. The cells were rinsed with water and air-dried. For Alizarin red staining, the cells cultured for 14 days in the osteoblast differentiation medium were washed twice with PBS, fixed in 3.7% formaldehyde for 10 min, and then stained with 0.1% Alizarin red (Sigma–Aldrich) at pH 6.3 for 10 min. For calcium measurement, cell lysates from the cells cultured for 21 days in the osteoblast differentiation medium were collected in lysate buffer (100 mM Tris–HCl, pH 7.5) and used for calcium measurement by using the calcium assay kit (Cayman, MI). According to the manufacturer, the reaction was measured spectrophotometrically at 590 nm. Calcium content was normalized to protein content measured by Protein Assay Reagent (Bio–Rad).

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChIP assay was carried out using previously described procedures [Fei et al., 2010]. Briefly, the shCont and shUtx cells were cultured for 3 days in the osteoblast differentiation medium and chemically cross-linked with 1% formaldehyde for 15 min at ambient temperature. Cells were lysed, sonicated, and immunoprecipitated with 4 μg antibodies pre-absorbed with 40 μl protein A/G beads overnight at 4°C. After several washes, the complexes were eluted and the cross-linking was reversed by overnight incubation at 65°C. Extracted (input) and immunoprecipitated DNA was then purified by the treatment with RNase A, proteinase K, and multiple of phenol: chloroform: isoamyl alcohol. Real-time PCR was performed with the primers corresponded to the promoter regions of Runx2 and Osterix as follows:

Runx2 promoter primers:
forward, 5′-AAGGAGTTTGCAAGCAGAGC-3′,
reverse, 5′-CAACTGAGTGTGTGGCGTTC-3′;
Osterix promoter primers:
forward, 5′-GAAGCTCTGACAACTTGCCC-3′,
reverse, 5′-AAGGGAGAGGGAGGGAGAAT-3′

STATISTICAL ANALYSIS

Each series of experiments were repeated at least three times and all the data were expressed as mean values ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test when needed to analyze data between two or more groups. Statistical significances were indicated with (**P* value < 0.05) or (***P* value < 0.01). A *P* value < 0.05 is considered significantly different.

RESULTS

EXPRESSION OF Utx INCREASED DURING OSTEOBLAST DIFFERENTIATION

MC3T3-E1 cells were cultured in the osteoblast differentiation medium for the indicated periods and the expression levels of Utx were examined. Figure 1A shows that Utx expression increased during osteoblast differentiation in a time-dependent manner, as determined by real-time PCR. Western blot analysis also revealed that the protein level of Utx increased during osteoblast differentiation (Fig. 1B). The levels of β-actin, used as the internal control, did not change during the periods (Fig. 1B). The similar expression patterns of Utx mRNA (Fig. 1C) and protein (Fig. 1D) were observed in primary osteoblasts during osteoblast differentiation.

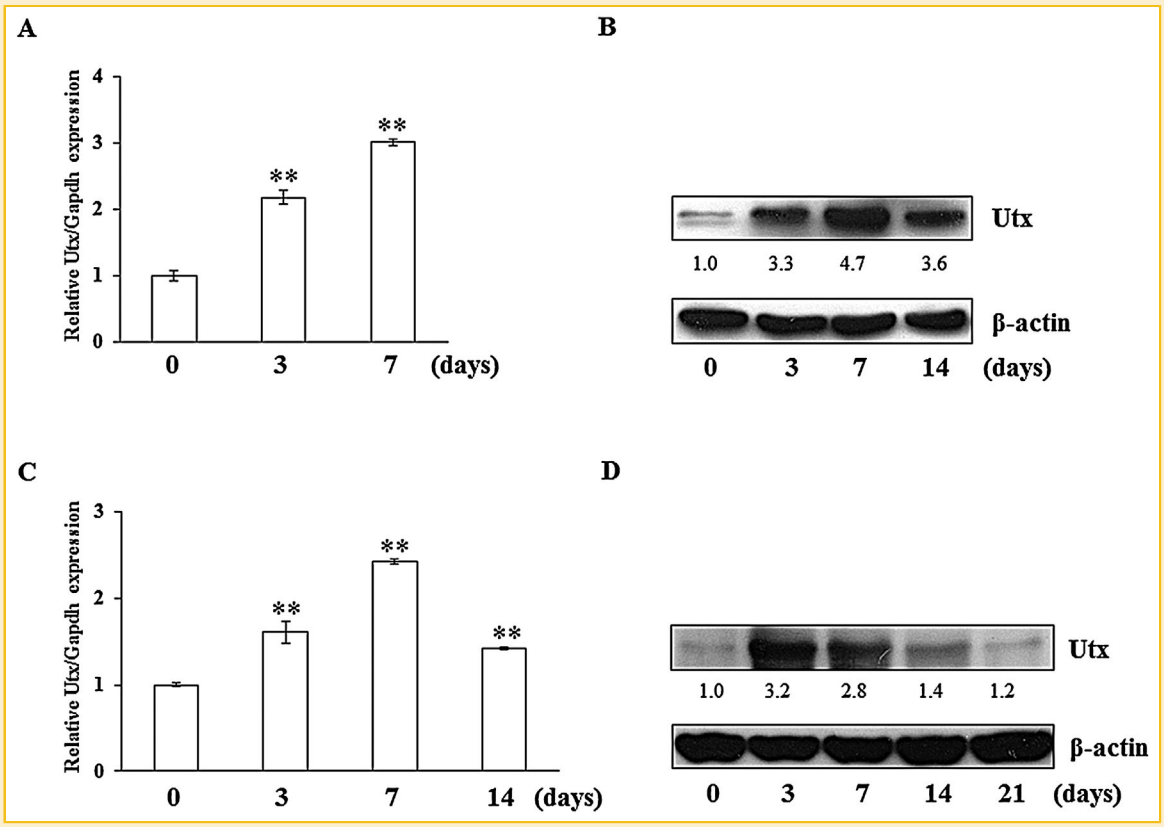

Fig. 1. Expression of Utx increased during osteoblast differentiation. MC3T3-E1 cells and primary osteoblasts were cultured in the osteoblast differentiation medium for the indicated periods. Utx expression was examined by real-time PCR (A) and Western blot analysis (B) in MC3T3-E1 cells. The mRNA (C) and protein levels (D) of Utx were also examined in primary osteoblasts. All the data of the real-time PCR are presented as means ± SEM of representative analysis from three separate experiments. $^{**}P < 0.01$. Utx protein levels were quantified by Image J, normalized to β-actin levels and compared with the 0 day control.

INHIBITION OF Utx DEMETHYLASE ACTIVITY IMPAIRED OSTEOBLAST DIFFERENTIATION

To evaluate whether demethylase activity of Utx is involved in osteoblast differentiation, MC3T3-E1 cells in the osteoblast differentiation medium were treated with GSK-J1, a potent inhibitor of H3K27me3-specific demethylase. GSK-J1 suppressed the expressions of Runx2 and Osterix as determined by real-time PCR (Fig. 2A). We used 10 μM GSK-J1 in the following experiments. Treatment of GSK-J1 increased the global levels of H3K27me3 in MC3T3-E1 cells, whereas the levels of H3K4me3, H3K9me3, and H3K36me3 were not affected (Fig. 2B). Histone H3 level was not changed with GSK-J1-treatment. Accumulation of ALP was suppressed in the cells treated with GSK-J1 (Fig. 2C). GSK-J1 also decreased the ALP activity in MC3T3-E1 cells (Fig. 2D).

STABLE SILENCING OF Utx EXPRESSION IMPAIRED OSTEOBLAST DIFFERENTIATION AND MINERALIZATION

To further examine the roles of Utx in osteoblast differentiation, we established several stable Utx knockdown cells (♯1, ♯2, ♯3) by infecting the lentivirus expressing Utx-specific shRNA (shUtx). MC3T3-E1 cells infected with nonspecific shRNA were used as a control (shCont). The expression of Utx significantly decreased in the shUtx cells compared with that in the shCont cells, as determined by real-time PCR (Fig. 3A). Knockdown of Utx increased the level of H3K27me3, but did not affect the level of H3K4me3 (Fig. 3B). There are no differences in the rate of cell proliferation in shCont and shUtx cells (data not shown). The shCont and shUtx cells were cultured in the osteoblast differentiation medium and ALP activity and mineralization were assessed by ALP, Von Kossa, and Alizarin red staining. The intensity of these staining decreased in the shUtx cells compared with that in the shCont cells (Fig. 3C). In accordance with the staining results, ALP activity was significantly lower in the lysate from the shUtx cells compared with that of the shCont cells (Fig. 3D). Calcium level was also significantly lower in the shUtx cells (Fig. 3E). To investigate the molecular mechanism of the impaired osteoblast differentiation in shUtx cells, the expression of bone-related genes were examined by real-time PCR. As shown in Figure 3F, the expressions of Runx2, Osterix, OCN, OPN, and BSP decreased in the shUtx cells compared with those in the shCont cells.

SILENCING OF Utx DECREASED RUNX2 AND OSTERIX PROMOTER ACTIVITIES AND INCREASED THE LEVEL OF H3K27me3 ON THE PROMOTER REGIONS OF RUNX2 AND OSTERIX

To study the mechanisms responsible for the suppression of osteoblast differentiation in shUtx cells, we examined the promoter activities of transcription factors Runx2 and Osterix. The luciferase

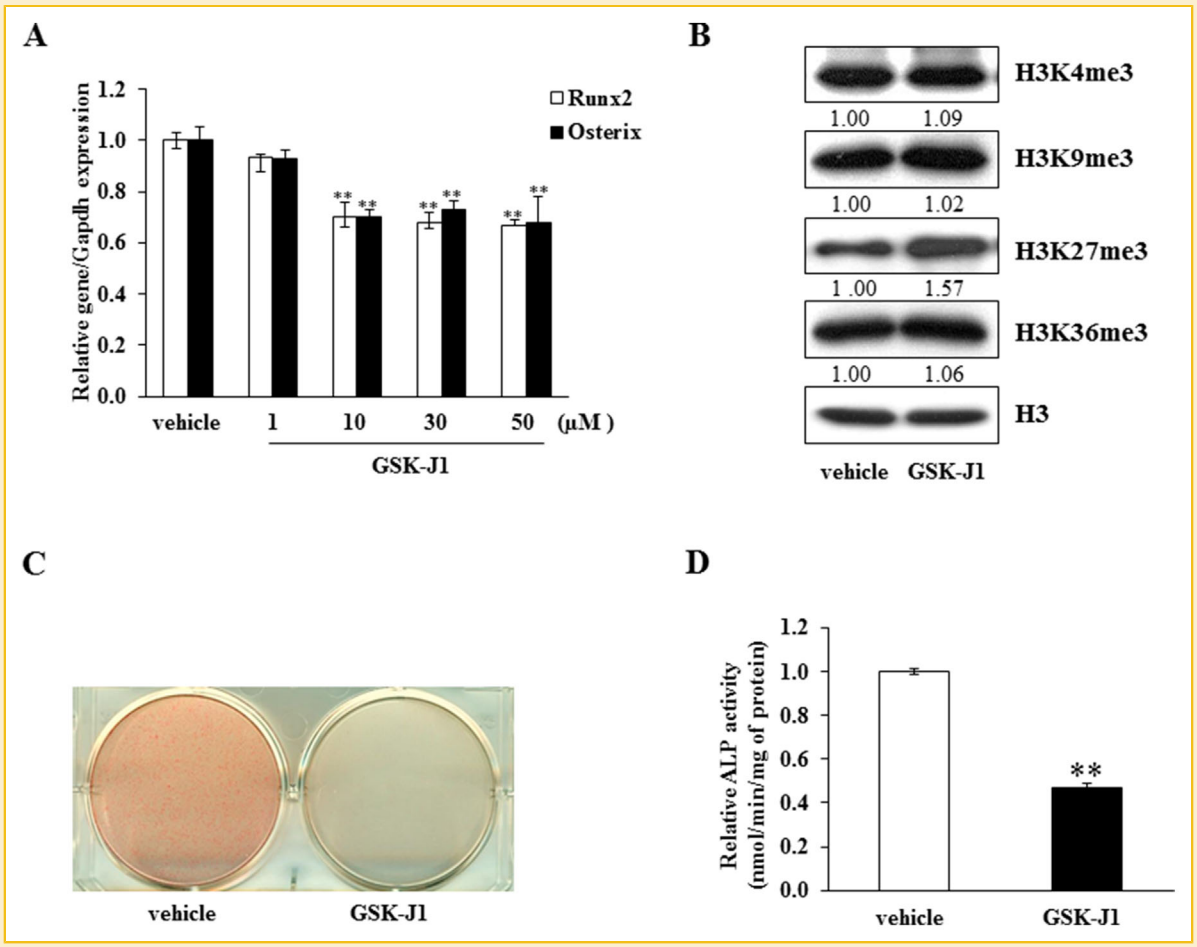

Fig. 2. GSK-J1 suppressed osteoblast differentiation. MC3T3-E1 cells were pretreated with GSK-J1 for 2 h and then cultured in the osteoblast differentiation for 3 days (for RNA and protein extraction) or 7days (for ALP staining and ALP activity). (A), RNA was extracted and real-time PCR was performed for Runx2 and Osterix. Values represent the mean ± SEM of representative analysis from three separate experiments. (B), Cell lysates were collected and subjected to Western blots analysis using the indicated antibodies. The protein levels of H3K4me3, H3K9me3, H3K27me3, and H3K36me3 were quantified by Image J, normalized to H3 levels, and compared with the vehicle control. (C), Cells cultured for 7 days in the osteoblast differentiation medium were fixed and stained for ALP accumulation. (D), Cells cultured for 7 days in the osteoblast differentiation medium, cell lysates were collected and ALP activity was measured. $^{**}P < 0.01$.

activities of Runx2 (Fig. 4A) and Osterix (Fig. 4B) promoters were decreased in the shUtx cells. To assess whether the decreased activity could be resulted from the changes of histone modification on the promoter region, ChIP assay was performed using anti-H3K27me3 and anti-H3K4me3 antibodies. Knockdown of Utx increased the levels of H3K27me3 on the Runx2 (Fig. 4C) and Osterix (Fig. 4D) promoter regions, but did not significantly affect the levels of H3K4me3 (Fig. 4E and 4F).

DISCUSSION

Dynamic changes in posttranslational histone modification are closely linked with gene expression by relaxing or compressing the chromatin structure to allow or reject transcription factors to access to the target DNA sequences. A growing number of studies have shown that epigenetic changes on the specific regions of histone protein including H3K27me3 reflect the determination of cell lineage commitment. In this study, we examined the expression and function of H3K27me3-specific demethylase Utx in osteoblast differentiation.

Recently, we have demonstrated that Jmjd3 regulates osteoblast differentiation via Runx2 and Osterix [Yang et al., 2013]. Since Utx is a closely related family member of Jmjd3, we hypothesized that Utx could be also involved in osteoblast differentiation. In the present study, we demonstrated that Utx expression increased in the early stages during osteoblast differentiation in MC3T3-E1 cells and primary osteoblasts, while the level of H3K27me3 decreased during this process [Yang et al., 2013]. Inhibition of Utx activity by GSK-J1 increased the level of H3K27me3, which is consistent with the previous report [Kruidenier et al., 2012], and suppressed Runx2 and Osterix expressions and ALP activity. These results imply that Utx activity is involved in osteoblast differentiation through regulating the expression of bone-related genes including Runx2 and Osterix.

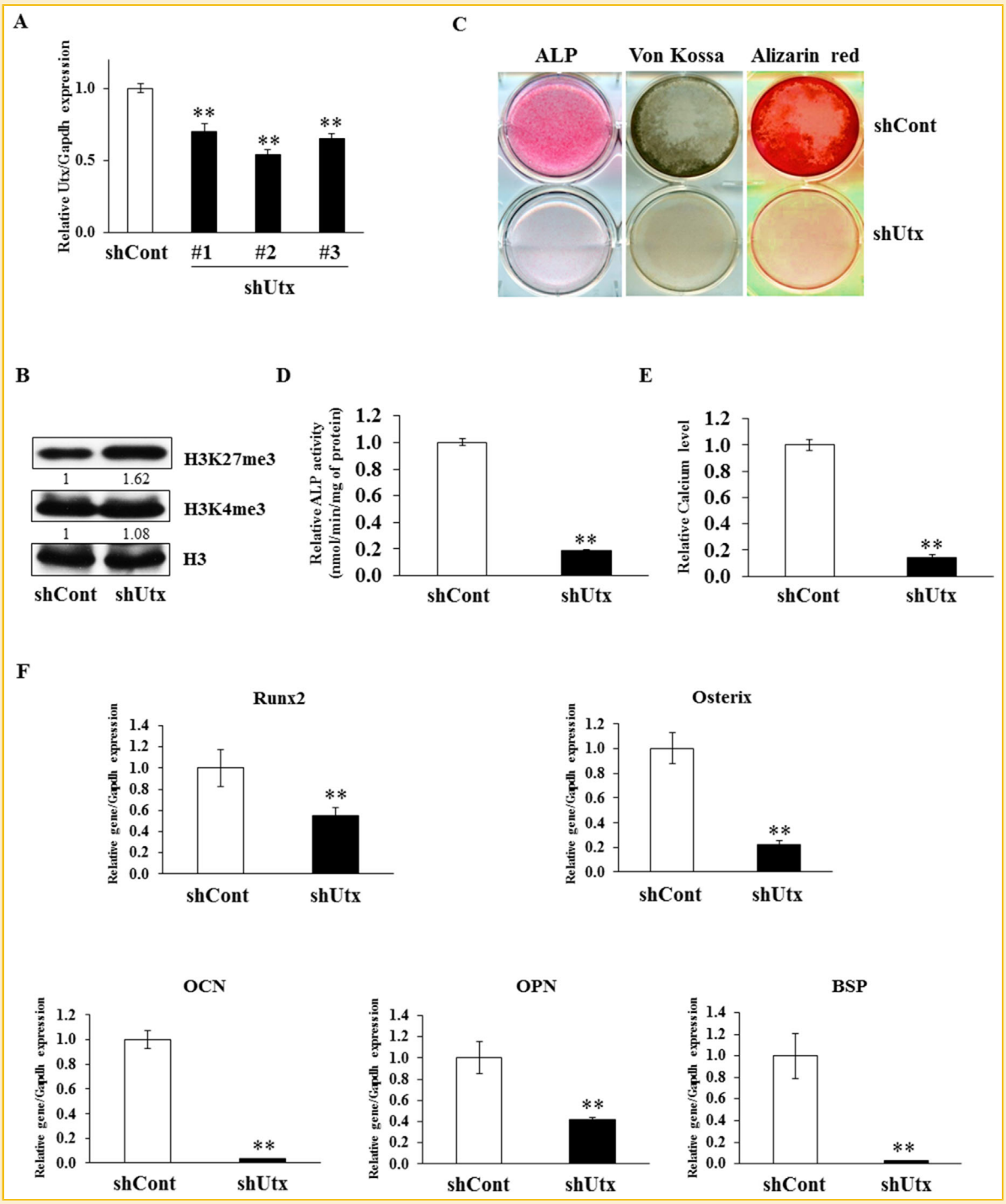

Fig. 3. Stable silencing of Utx suppressed osteoblast differentiation. (A), MC3T3-E1 cells were transfected with control (shCont) or Utx-specific shRNA (shUtx) and the stable knockdown cells were constructed. RNA was extracted and real-time PCR was performed for Utx. (B), Cell lysates were collected and subjected to Western blot analysis using the indicated antibodies. (C), The shCont and shUtx cells were cultured in the osteoblast differentiation medium for 7 days (ALP activity) or 14 days (Von Kossa and Alizarin red). The cells were stained for ALP activity (left panel), and mineralization by Von Kossa (central panel) and Alizarin red (right panel). (D), The shCont and shUtx cells were cultured for 7 days in the osteoblast differentiation medium and ALP activity was measured. (E), The shCont and shUtx cells were cultured for 21 days in the osteoblast differentiation medium and the calcium level in the cultured cells were quantified. (F), The shCont and shUtx cells were cultured for 3 days. RNA was extracted from the cells and the mRNA levels of bone markers were determined by real-time PCR with normalization by Gapdh expression. Each bar represents the mean ± SEM of representative analysis from three separate experiments. $^{**}P < 0.01$.

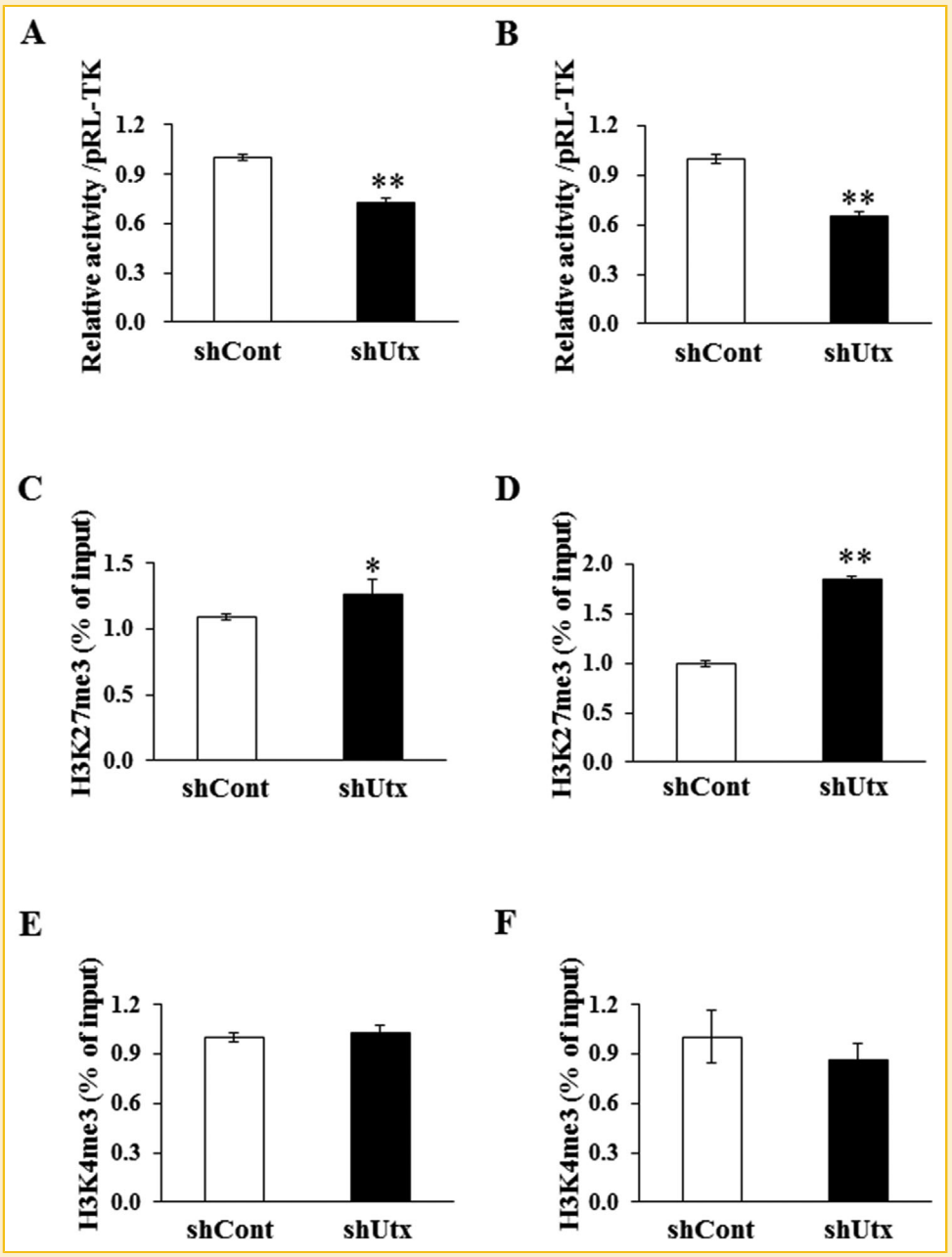

Fig. 4. Silencing of Utx decreased the promoter activities of Runx2 and Osterix and increased the level of H3K27me3 on the Runx2 and Osterix promoter regions. (A, B), Cells were transfected with the luciferase construct containing Runx2 (A) or Osterix (B) promoter regions and then cultured in osteoblast differentiation medium for 24 h. Cell lysates were collected and then the luciferase activity was measured. (C–F), Cells were cultured in osteoblast differentiation medium for 3 days. ChIP analysis were performed to examine the levels of H3K27me3 on the Runx2 (C) or Osterix (D) promoter regions and the levels of H3K4me3 on the Runx2 (E) or Osterix (F) promoter regions. Data are expressed relative to the value of sample from the control cells and values represent the mean ± SEM of representative analysis from three separate experiments. *$P < 0.05$, **$P < 0.01$.

As GSK-J1 is an inhibitor of both Jmjd3 and Utx, we could not exclude that the inhibited activity of Jmjd3 might also contribute to the suppressed osteoblast differentiation by GSK-J1 treatment. Reduction of Utx by shRNA suppressed osteoblast differentiation accompanied with decreased expressions of bone-related genes including Runx2, Osterix, OCN, OPN, and BSP. These gene expressions were also inhibited in the Utx-knockdown cells stimulated with bone morphogenetic protein-2 (data not shown). These results suggest that Utx is a positive regulator of osteoblast differentiation and bone-related gene expressions.

The H3K27me3 demethylases remove a repressive methyl mark and then increase the promoter activity by inducing a generalized remodeling of the locus. Since Utx was reported to orchestrate the chromatin signature of gene promoters [Cho et al., 2007; Lee et al.,

2007], we tried to determine whether Utx plays a direct role in transcriptional activation on Runx2 and Osterix promoter regions. Knockdown of Utx decreased the promoter activities of Runx2 and Osterix and increased the levels of H3K27me3 on the Runx2 and Osterix promoters. These results suggest that Utx controls the promoter activities of these genes through the modification of H3K27 methylation status. Runx2 and Osterix are essential transcription factors for osteoblast differentiation by inducing the expressions of OCN, OPN, and BSP, which are required for the terminal osteoblast mineralization and bone formation [Nakashima et al., 2002; Hill et al., 2005; Komori, 2006]. Our present results suggest that decreased expression of Runx2 and Osterix in the shUtx cells leads to the downregulation of other bone-related genes, resulting in the impaired osteoblast differentiation and mineralization.

The data about Utx in this study is in line with the recent publication showing that epigenetic switch of methylation status on H3K27 elicits the determination of human mesenchymal stem cell (MSC) lineage [Hemming et al., 2014]. In their report, they demonstrated that H3K27 methyltransferase Ezh2 was a negative regulator of osteogenesis, while Utx was found to promote the osteogenic commitment of MSCs. Utx is located at Xp11.2 on X chromosome, but escapes X-chromosome inactivation [Hübner and Spector, 2010]. Recently, it was reported that Utx is a gender specific tumor suppressor [Van der Meulen et al., 2015]. Further study is needed to clarify whether Utx regulates osteoblast differentiation and bone formation in a gender specific way or not.

Besides H3K27me3, lysine methylation results in the unique transcriptional outcomes depending on the methylation sites. Trimethylation of histone H3 at lysine-4, -36, and -79 (H3K4, H3K36, and H3K79) are implicated in the transcriptional activation, whereas trimethylated H3K9 and H4K20 as well as H3K27 are considered as hallmarks of transcriptional repression [Margueron et al., 2005]. Accumulating evidence has shown that alteration of methylation status modifies chromatin structure and is involved in osteoblast differentiation. NO66, a Jumonji C-domain-dependent histone demethylase specific for H3K4 and H3K36, directly interacts with Osterix and regulates Osterix-target genes in osteoblasts [Sinha et al., 2010]. SETDB1, a histone methylransferase for histone H3K9, associated with peroxisome proliferator-activated receptor γ (PPARγ) to methylate H3K9 in the PPARγ-targeted genes to induce osteoblastogenesis by suppressing adipogenesis in bone marrow stem cells [Takada et al., 2007]. Our present study does not exclude the involvement of these methylases and demethylases in osteoblast differentiation.

Our previous and present studies showed that both Utx and Jmjd3 regulate osteoblast differentiation through the modification of methylation status of H3K27 on the promoter regions of Runx2 and Osterix. Jmjd3 partly compensates for the loss of Utx during the differentiation of embryonic stem cells [Morales Torres et al., 2013], but major change in Jmjd3 expression was not observed in the Utx knockdown cells (data not shown). In addition to the role as a H3K27me3 demethylase, Utx might be involved in the methylation of H3K4 by supporting methyltransferase MLL3/MLL4 for H3K4me3 [Vandamme et al., 2012]. Indeed, the tendency of decreased level of H3K4me3 was observed on the promoter region of Osterix in the Utx knockdown cells. We could not completely exclude the possibility that Utx also regulates osteoblast differentiation through a different mechanism from Jmjd3. Although more studies are required to determine how Utx and Jmjd3 work cooperatively and/or independently to regulate gene expression, it would be helpful to understand the epigenetic regulation in osteoblast differentiation.

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