An HDAC Inhibitor, Entinostat/MS-275, Partially Prevents Delayed Cranial Suture Closure in Heterozygous Runx2 Null Mice

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ABSTRACT
Cleidocranial dysplasia (CCD) is an autosomal dominant skeletal disorder caused by mutations in RUNX2, coding a key transcription factor of early osteogenesis. CCD patients suffer from developmental defects in cranial bones. Despite numerous investigations and clinical approaches, no therapeutic strategy has been suggested to prevent CCD. Here, we show that fetal administration of Entinostat/MS-275, a class I histone deacetylase (HDAC)-specific inhibitor, partially prevents delayed closure of cranial sutures in Runx2¹⁺² mice strain of C57BL/6J by two mechanisms: 1) posttranslational acetylation of Runx2 protein, which stabilized the protein and activated its transcriptional activity; and 2) epigenetic regulation of Runx2 and other bone marker genes. Moreover, we show that MS-275 stimulates osteoblast proliferation effectively both in vivo and in vitro, suggesting that delayed skeletal development in CCD is closely related to the decreased number of progenitor cells as well as the delayed osteogenic differentiation. These findings provide the potential benefits of the therapeutic strategy using MS-275 to prevent CCD. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: CLEIDOCRANIAL DYSPLASIA; MS-275; HDI; RUNX2; SKELETOGENESIS

Introduction

RUNX2, the central transcriptional factor of osteogenesis, regulates osteogenic cell proliferation and differentiation.¹,² In concert with other osteogenic transcription factors, including Osterix (Ox), Dlx5, and NFIB–C, RUNX2 controls the functions of osteoblasts to regulate expression of bone marker genes such as osteocalcin.³,⁴ Mouse fetuses harboring targeted disruption of Runx2 exhibit a complete lack of ossification due to maturation arrest of osteoblasts, indicating that this gene plays an indispensable role at the early stages of osteoblast differentiation.¹,⁵ The biological and clinical significance of RUNX2 was established by the identification of a mutation in the RUNX2 gene locus in humans with cleidocranial dysplasia (CCD), an autosomal dominant skeletal disease.⁶,⁷ The symptoms of CCD include claviculare hypoplasia, delayed development, and ossification of cranial bones. Runx2¹⁺² mice exhibit symptoms similar to those of human CCD patients,⁶,⁷ indicating the importance of RUNX2 in intramembranous bone formation.

Previously, Lou and colleagues⁸ established a mouse model in which Runx2 expression can be finely regulated by neo cassette. They found that the Runx2 mRNA level must drop below a critical threshold for CCD-like phenotypes to develop; mice expressing lower levels of Runx2 mRNA (55% to 70% of that in the wild type [WT]) developed CCD-like phenotypes, whereas mice with higher Runx2 mRNA levels (>79% of WT) developed a normal skeleton. These findings prompted us to hypothesize that symptoms of CCD could be alleviated when functional Runx2 transcriptional activity is restored to the threshold level.

Many osteogenic signals, such as BMP2, TGF-β, FGF2, and PTH, regulate Runx2 expression during the early stages of osteogenesis.⁹ However, because of their nonspecific effects on overall biological processes, it would be challenging to use these
growth factors for human treatment. In previous works, we showed that the stability and transactivation activity of RUNX2 protein are regulated by serial posttranslational modifications such as phosphorylation, prolyl isomerization, acetylation, and ubiquitination. Accordingly, modulation of one or more of these posttranslational modifications of RUNX2 represents a therapeutic target for treatment of the genetic disease CCD.

Among the posttranslational modifications of RUNX2, acetylation of several lysine residues in RUNX2 C-terminus is necessary to stabilize and activate the protein. Numerous in vitro studies illustrated that HDAC inhibitors (HDIs) induce acetylation of both histone and non-histone proteins, including RUNX2. In addition, our previous studies showed that MS-275, an HDI, exerts a strong bone anabolic effect in models of calvarial defect or osteoporosis in adult animals. MS-275 is also known clinically as Entinostat (Syndax Pharmaceuticals Inc., Waltham, MA, USA) and currently being used in clinical trials for treating cancers. Based on these previous results, we hypothesize that MS-275 also exerts a RUNX2-mediated anabolic effect in the early stage of bone development in fetal animals. In this study, we tested our hypothesis by administering MS-275 to RUNX2-haplodeficient mice, a model for human CCD, and observed the effects of the drug on their developmental bone defects.

Materials and Methods

Animal experiments

RUNX2-deficient mice were derived as described previously and maintained under specific pathogen-free conditions with autoclaved diet (Altamin, Eastern-Westphalia, Germany). WT and RUNX2+/− mice were mated to obtain littermates having equal proportions of both genotypes. Eight to 14-week-old pregnant mice were intraperitoneally injected with MS-275 (Calbiochem, Darmstadt, Germany). We chose mice randomly to perform each experiment. Skeletal staining and microCT analysis were performed as previously described. The number of animals in each experimental group is indicated in Supplemental Table S4. Animals were weaned in mouse gang cages following IACUC policies. All animal studies were reviewed and approved by the Special Committee on Animal Welfare, Seoul National University, Seoul, Republic of Korea (approval no. SNU-20160512-3).

Double fluorescence labeling and calvarial organ culture of calvaria

Fluorescent labeling for determination of new bone growth in organ culture was described previously. Pregnant mice were injected intraperitoneally with 1 mg/mL calcine (Sigma-Aldrich, St. Louis, MO, USA) solution at 14.5 and 16.5 days post-coitum (dpc). Calvaria of E17.5 were cultured for 48 hours in osteogenic media supplemented with 0.09 mg/mL Alizarin complexone (Sigma-Aldrich), with or without 1 μM MS-275. Harvested explant were processed for tissue analysis and observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Cell culture and cell proliferation assay

WT and RUNX2+/− mouse calvarial cells were isolated from E18.5 mice calvaria and cultured in α-MEM with 10% fetal bovine serum (FBS) containing 1% antibiotics. Osteogenic media includes 5 mM β-glycerophosphate and 50 μg/mL ascorbic acid. Viable cell number was measured by water-soluble tetrazolium assay using EZ-CyTox solution (Daeil Lab Service, Seoul, Korea).

Immunohistassays

Detection of cellular protein by immunohistochemistry was performed as described previously. Immunohistochemical analyses were performed using the Dako Cytomation Envision System (Dako, Glostrup, Denmark). Stained tissues were visualized on a conventional microscope equipped with a DP72 digital camera (Olympus, Tokyo, Japan). Antibodies used for these experiments are listed in Supplemental Table S1.

Transactivation activity of RUNX2

Transacting activity of RUNX2 was measured using the G6OS2-Luc or rat osteocalcin (OC) promoter–Luc reporter plasmids, as described previously.

RNA-seq procedure and data analysis

Calvarial of E17.5 mice treated with MS-275 or vehicle at E14.5 and E16.5 in utero were extracted in biological triplicate and total RNA was isolated using the Qiagen reagent (Qiagen, Hilden, Germany). The mRNA in total RNA was converted into a library of template molecules suitable for cluster generation using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). The TruSeq RNA libraries were quantitated by qPCR. Using these RNA libraries, paired-end sequencing with 76 bp read length was performed on the NextSeq500 platform (Illumina) to generate FASTQ-formatted sequence data. Transcriptome levels were determined from the RNA-seq reads via the following RNA-seq pipeline. First, to remove sequencing artifacts and poor-quality bases, raw data quality control was conducted using Trimmomatic (v0.33) with the following options: PE --phred33 ILLUMINAACLIP:TruSeq3-PE:fa:2:30:10 MINLEN:50 Trailing:20. Reads were mapped to the mouse genome reference (MM10) from the Ensembl database using TopHat2 (v2.1.0). Conversion and sorting of the alignment file was performed using Samtools (v0.1.19). The HTSeq package was used to estimate the count of uniquely mapped reads for each of the 39,179 annotated genes in the gene transfer format (.GTF) file. From this RNA-seq analysis pipeline, we obtained the expression level of 39,179 genes from 12 samples. The edgeR tool was used to detect differentially expressed genes (DEGs), which were classified as either up- or downregulated depending on fold change. The DAVID Bioinformatics Resource 6.7 (NIAMD, NIH) was used for Gene Ontology (GO) pathway analyses.

Reverse transcription PCR and quantitative real-time PCR

Calvarial RNA samples from litter-matched WT and RUNX2+/− mice were reverse-transcribed with the PrimeScript RT kit (Takara Bio, Shiga, Japan). Quantitative real-time PCR for analysis of genes was performed as described previously. The list of primers is provided in Supplemental Table S3. All samples were run in duplicate.

Epigenetic landscape analysis

Experiments for epigenetic landscape analysis, methylation-specific PCR, ChIP assay, and in vitro methylation were described previously. EMBOS CpGPlot (http://www.ebi.ac.uk/emboss/cpgplot) was used to identify CpG islands. All experiments were

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performed with primary mouse calvarial cells. PCR primer pairs used in these assays and their targeting sites are illustrated in Supplemental Table S3 and Supplemental Fig. S3, respectively.

Statistics

All quantitative data are presented as means ± SD. Each experiment was performed at least three times. Statistical differences were analyzed by Student’s t test. \( p \leq 0.05 \) was considered to indicate a significant difference.

Results

MS-275 partially restored CCD-like phenotypes in Runx2\(^{-/-}\) mice

To determine whether MS-275 can functionally compensate for CCD-like phenotypes in Runx2-haploinsufficient mice, we established a single-injection regimen in which MS-275 was administered to pregnant mice. According to previous studies, dynamic skeletogenesis in cranial bones occurs between E14.5 and birth.\(^{11,12}\) Therefore, we administered MS-275 at 14.5 dpc and euthanized fetuses at 16.5 dpc. All fetuses were evaluated for their level of bone development using measurements of ossified vertebrae length, ratio of ossified area over whole skull area, and sizes of the mandible and clavicle (Fig. 1A–H). MS-275 treatment had an effect on overall skeletal growth. Ossified vertebral length was 40% shorter in Runx2\(^{-/-}\) than in their WT littermates, but MS-275 treatment of Runx2\(^{-/-}\) mice significantly increased their ossified vertebral lengths nearly to WT levels (Fig. 1A, B; \( p < 0.05 \)). Similar growth was observed in membranous bones, including calvaria, mandibles, and clavicles. MS-275 treatment of Runx2\(^{-/-}\) mice significantly (\( p < 0.05 \)) increased mineralization of these bones nearly to WT levels (Fig. 1C–H). In calvarial bone and mandible, the therapeutic effect of MS-275 was remarkable: Runx2\(^{+/+}\) traits were recovered almost to the WT level. By contrast, the drug had a relatively small effect on clavicle development, although the increase in mineralization was still significant (\( p < 0.05 \)) and the associated morphological change was meaningful (Supplemental Fig. S1).

To determine whether MS-275 treatment affects bone development until they are born, we examined calvarial phenotypes of pups at P0 after administration of MS-275 to pregnant mice at 14.5 dpc and 16.5 dpc considering the half-life of MS-275 (1 hour in rodents) and confirmed that it was effective. Under these treatment conditions, the CCD-like phenotypes of P0 Runx2\(^{+/-}\) mice were almost abolished, and the mice were nearly WT in appearance (Fig. 1I–K). In Fig. 1I, we observed that MS-275 also leads bone formation in the WT group. It is compatible with our previous research, which showed therapeutic value of MS-275 in osteoporosis-induced adult mice.\(^{13}\) In Supplemental Fig. S2, we showed no significant difference between the MS-275- and vehicle-treated group. Based on these data, it is clear that maintenance of an adequate therapeutic level of MS-275 is critical and that a well-controlled pharmacokinetic study of its effects should be performed.

To rule out maternal effects of MS-275, we employed a mouse calvarial organ culture system. In Fig. 1L, calcein labeling of calcified tissue in utero was observed as green fluorescence, and newly mineralized tissue formed during organ culture was observed as red fluorescence. From these observations, we could estimate growth through the effect of MS-275 treatment alone. MS-275 treatment caused a strong increase in newly generated calvarial bone in both genotypes of mice, but the recovery effect was more dramatic in Runx2\(^{+/-}\) than in WT mice (Fig. 1L). Thus, the distance between osteogenic fronts of parietal bone was less in the presence of MS-275. The results of coronal sectional view strongly support this observation (Fig. 1L–M). It is notable that MS-275 stimulates mineralization on the osteogenic front. Therefore, these results indicated that MS-275 is a positive regulator of intramembranous bone formation in vivo.

MS-275 restores Runx2 protein acetylation and stabilizes its transactivating activity in Runx2\(^{+/-}\) calvaria cells

We showed previously that HDI stabilizes Runx2 by posttranslational modification;\(^{11}\) however, it remains unclear whether it could compensate for Runx2 activity in Runx2-deficient organisms. To address this possibility, we examined the changes of acetylated Runx2 level in WT and Runx2\(^{+/-}\) calvarial cells after MS-275 treatment. Immunoprecipitation and immunoblot analysis showed that acetylation of Runx2 was lower in Runx2\(^{+/-}\) mouse calvarial cells than in WT cells. By contrast, acetylated Runx2 level of MS-275-treated Runx2\(^{+/-}\) mouse calvaria cells was restored to that of the WT-vehicle-treated group (Fig. 2A). To determine whether MS-275 increases the amount of Runx2 protein, we examined the endogenous Runx2 level. Indeed, the amount of Runx2 increased in a dose-dependent manner upon MS-275 treatment (Fig. 2B, C).

Next, we investigated whether MS-275 stabilizes the Runx2 protein. The degradation rate was lower in the MS-275-treated group than in the vehicle-treated group: specifically, the half-life of Runx2 was 13.02 hours in the absence of MS-275 and 18.15 hours in the presence of MS-275 (Fig. 2D–E). Moreover, immunofluorescence assays revealed that the total Runx2 protein level was elevated in both genotypes of MS-275-treated mouse calvarial cells. In the Runx2\(^{+/-}\) cells, the expression level of Runx2 protein in nucleus was restored close to WT levels (Fig. 2F, G). We observed similar Runx2 expression patterns in mouse calvarial tissue. The amount of Runx2 protein accumulated in the nucleus of osteoblasts around calvarial bone was elevated in MS-275-treated Runx2\(^{+/-}\) mouse calvaria (Fig. 2H). Because both acetylation and ubiquitination occur at similar lysine residues,\(^{10}\) this observation could be attributed to a protective mechanism that inhibits the ubiquitination and proteasomal degradation pathway.

To determine whether MS-275 affects Runx2 transactivating activity, we carried out reporter assays. The results show that MS-275 upregulated Runx2 transcription activity in mouse calvarial cells of both genotypes. Remarkably, a high dose of MS-275 restored Runx2 transactivating activity in Runx2\(^{+/-}\) cells to WT levels (Fig. 2J). Additionally, because osteocalcin is a direct target of Runx2,\(^{24}\) we investigated the role of MS-275 on Runx2-mediated promoter activity. Runx2 overexpression significantly activated osteocalcin (OC) promoter activity but not the activity of a mutant reporter gene with an altered Runx2-binding site. Runx2 overexpression and MS-275 treatment synergistically stimulated OC-promoter activity. On the other hand, Runx2 overexpression could not stimulate the mutant OC-promoter with the altered Runx2 binding-site mutant, whereas MS-275 could still stimulate the mutant construct (Fig. 2J). Based on these observations, we hypothesize that MS-275 might influence not only Runx2 but also other transcription factors that bind in that region. Taken together, these results suggest that
MS-275 enhances transcription activity of Runx2 via posttranslational acetylation of Runx2.

MS-275 induces epigenetic regulation of the Runx2 promoter region

Because MS-275 is a histone modulator, we hypothesize that treatment of MS-275 would change the epigenetic landscape of Runx2 promoter in primary calvarial cells. Runx2 has at least two different isoforms using alternative promoters, which show differential expression patterns in mouse calvaria development. P1 promoter (distal) drives transcription of type II Runx2 (Runx2-II) known for bone-specific expression, whereas P2 promoter (proximal) induces type I Runx2 (Runx2-I) expression in undifferentiated mesenchymal stem cell, preosteoblasts and chondrocyte precursors. In silico analysis predicted only one CpG island in Runx2 gene, which is located near the transcription start site in the Runx2-P2 promoter. We targeted that region to measure Runx2 expression and observed changes in several epigenetic markers (Supplemental Fig. S3). First, we confirmed that Runx2-I expression levels in primary mouse calvarial cells were increased by MS-275 treatment (Fig. 3A). To investigate whether MS-275 has an epigenetic function, we performed chromatin immunoprecipitation (ChIP) assays (Fig. 3B). We checked the levels of acetylated histone H4 (Ac-H4), dimethylated histone H3K9 (Me-H3K9), and methyl CpG binding protein 2 (MeCP2). The level of Ac-H4, a hallmark of transcriptionally activated genes, was increased by MS-275 treatment. By contrast, the levels of Me-H3K9 and MeCP2 binding, both of which are markers of epigenetic suppression, decreased after MS-275 treatment (Fig. 3B). The results of the ChIP assay revealed genomic interactions of epigenetic factors with the Runx2 promoter region; these

Fig. 1. MS-275 rescues the CCD-like phenotypes in Runx2<sup>−/−</sup> mice. Fetuses were treated with MS-275 or vehicle either once at 14.5 dpc or twice at 14.5 and 16.5 dpc in utero. (A) Representative skeletal staining of E16.5 WT and Runx2<sup>−/−</sup> mice that received one injection in utero. Scale bar = 5 mm. (B) Lengths of ossified spinal bone. The same samples were cut into (C) calvaria (scale bar = 2 mm), (D) mandible (scale bar = 2 mm), and (E) clavicles (scale bar = 1 mm). (F–H) Quantitative analysis of each sample is depicted as scatter-plot graphs to the right of each figure. Developing clavicles exhibit a significant increase in mineralized area. (I) Representative microCTs of skulls from newborn WT and Runx2<sup>−/−</sup> mice that received the indicated chemicals at E14.5 and E16.5. The asterisk (*) indicates rescue of CCD-like phenotype on calvaria. Ossification level of each group was measured as (J) % of ossified area over full skull area and (K) width between parietal bones. (L) Double fluorescence reveals new bone growth in organ-cultured E17.5 calvarial tissue. The tissue was visualized by fluorescence microscopy as whole tissue (upper) or coronal sections (lower) (scale bar = 1 mm). (M) Suture width of (L) was measured. Quantitative data were obtained using the ImageJ software. Veh. = vehicle; MS. = MS-275. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test.
Fig. 2. MS-275 stabilizes and activates Runx2 protein. (A) WT and Runx2<sup>−/−</sup> mouse calvaria cells were cultured in the presence or absence of 1 μM MS-275 for 24 hours. Acetylated Runx2 was measured by immunoprecipitation (IP). Because amount of Runx2 in Runx2<sup>−/−</sup> is almost half of that in WT, equal amounts of Runx2 inputs were estimated and normalized before IP. (B) Primary osteoblasts were cultured with MS-275 for 24 hours, and the abundance of endogenous Runx2 protein was examined by IB. (C) Protein band intensities in (B). (D) After transfection with Myc-tagged Runx2, primary calvaria cells were treated with or without 1 μM MS-275 for 24 hours and cultured with cycloheximide (CHX, 10 μg/mL) for the indicated times. Levels of Runx2 were assessed by detecting Myc protein. (E) Estimated change of Runx2 expression level over time. (F) Runx2 in each genotype of calvarial cells was detected after treatment with 1 μM MS-275 for 24 hours (scale bar = 10 μm). (G) Number of nuclear Runx2 foci in (F). (H) Runx2 levels in P0 mice calvarial tissue were determined by immunohistochemistry. Arrows indicate Runx2-positive osteoblasts. (Ha–d) show enlargement of the boxed regions. (I) Runx2-transactivating activity after 24-hour treatment of calvarial cells with the indicated dose of MS-275, assessed by luciferase assay using the 6XOSE2 reporter gene. The red line indicates the activity of vehicle-treated WT. (J) HEK293T cells were transfected with WT (-208 OC-Luc) or Runx2-binding site mutant osteocalcin (OC) promoter–Luc (-208 RUNX-mut) with or without Runx2 overexpression. Luciferase activity was measured after treatment with 3 μM MS-275 or vehicle for 24 hours. Quantitative data were obtained using ImageJ. Veh. = vehicle; MS = MS-275; E.V. = Empty vector. *p < 0.05, **p < 0.01, ***p < 0.001, #p < 0.0001, Student’s t test.
Fig. 3. MS-275 enables epigenetic modification of the Runx2 P2 promoter, resulting in elevated expression. (A–C) WT and Runx2−/− primary osteoblasts were treated with vehicle or 3 μM MS-275 for 24 hours. (A) Runx2-I levels were determined by qPCR. (B) Chromatin immunoprecipitation (ChIP) assays were performed using antibodies against H4 Ac, H3K9 Me, and MeCP. Chromatin fragments were PCR-amplified with primers for Runx2. (C) Methylation-specific PCR (MSP) analysis of the Runx2 promoter region. M and U represent amplification of methylated and unmethylated alleles, respectively. Bottom panel: Quantitation of MSP band density for unmethylated genes (dark gray bar) and methylated genes (light gray bar). (D) Digested or non-digested bands of Runx2 P2 promoter luciferase reporter vector after HpyCH4IV endonuclease treatment to confirm in vitro hypermethylation status. Methylation on the cytosine residue was carried by M.SssI CpG methyltransferase. (E–F) Runx2 promoter activity was assessed by luciferase reporter activity of (E) unmethylated promoter construct and (F) hypermethylated promoter. WT mouse calvarial cells were transfected with a Runx2 P2 promoter reporter construct, followed by the indicated dose of MS-275 treatment for 24 hours. Veh. = vehicle; MS. = MS-275. *p < 0.05, **p < 0.01, Student’s t test.

Interactions finely reflect the trend of Runx2 expression as shown in Fig. 3A.

Next, we carried out methylation-specific PCR to measure the DNA methylation status of the Runx2 promoter. Usually, hypermethylated DNA indicates a transcriptionally inactivated state.227 We found that MS-275 treatment decreased the amount of methylated PCR products and increased the amount of unmethylated DNA fragments in primary calvarial cells. Thus, MS-275 causes hypomethylation of 5′-cytosine in CpG islands of the Runx2 P2 promoter (Fig. 3C). Runx2-I gene expression (Fig. 3A) was inversely correlated with the methylation level of the CpG island in Runx2 P2 promoter (Fig. 3C). This result also suggested that Runx2 was transcriptionally activated by MS-275. To confirm the ability of MS-275 to activate the Runx2 gene, we performed an artificial in vitro methylation assay using M.SssI methyltransferase, as previously described.228 The HpyCH4IV restriction enzyme was used to validate promoter construct methylation: This enzyme recognizes the sequence ACGT and cuts selectively at sites containing unmethylated cytosine but cannot cut at CpG sites methylated by M.SssI (Fig. 3D). To assess the functional effects of MS-275 on CpG-methylated gene expression, we analyzed Runx2 promoter activity in luciferase reporter assays using in vitro methylated or unmethylated constructs. We found that a construct inactivated by CpG methylation was activated by MS-275 in a dose-dependent manner (Fig. 3E). These results also correspond with patterns of Runx2-I expression (Fig. 3A) and CpG methylation (Fig. 3C). Altogether, these in vitro results suggest that MS-275 induced Runx2 expression by affecting the epigenetic landscape of the Runx2 promoter region, which might increase the potential of primary calvarial cells to develop into mature osteoblasts.
MS-275 accelerates osteoblast differentiation and proliferation

As we showed in Fig. 2, MS-275 induced Runx2 acetylation, thereby generating the activated form of Runx2, which positively regulates cell proliferation and differentiation. Previously, we showed that proliferation of osteoblast precursor cells in osteogenic front regions is a critical mechanism underlying suture closure. Furthermore, several reports suggest that suppression of HDAC1 increases cell proliferation and global protein synthesis in osseous cells. Because MS-275 is a member of selective class I HDAC inhibitors, we expected that treatment of MS-275 would improve proliferative activity of calvarial cells and affect osteoblast differentiation. Therefore, we performed RNA-seq to investigate the action of MS-275 on fetal calvaria and to assess their transcriptomic response. GO term analyses revealed that administration of MS-275 affected various biological processes (Fig. 4A). Enrichment for GO terms associated with chromatin modification, skeletal development,
and regulation of proliferation support previously characterized roles of MS-275. The Venn diagrams shown in Fig. 4B show 4127 upregulated genes and 6057 downregulated genes (95% confidence, Student’s t test), of which 430 upregulated and 218 downregulated genes overlapped between E17.5 WT and Runx2/−/− calvaria. Table 1 shows representative hallmark genes, grouped according to biological process. In general, genes related to bone formation and proliferation were upregulated, whereas genes related to apoptosis were downregulated. First, we examined the in vitro effects of MS-275 on process of osteoblast differentiation. Expression levels of bone marker genes were elevated, indicating that MS-275 treatment rescued gene expression in Runx2/−/− cells (Fig. 4C). We investigated the early stages of osteoblast differentiation using ALP staining. MS-275 potentiated ALP activity in a dose-dependent manner in both WT and Runx2/−/− cells. ALP staining levels were lower in Runx2/−/− cells than in WT cells but were increased by a high dose of MS-275: Specifically, the staining level in Runx2/−/− cells exposed to 6 μM MS-275 were similar to those of vehicle-treated WT cells (Fig. 4D). In accordance with the elevated ALP activity, MS-275 also increased calcium deposition, as determined by Alizarin red S staining, which can show the late stages of osteoblast differentiation (Fig. 4E). These results are consistent with our assumption that MS-275 would compensate for the delayed differentiation potency of Runx2/−/− cells.

Next, we examined the effects of MS-275 on cell proliferation. Expression levels of proliferation marker genes were slightly elevated, as shown in Table 1. Notably, expression of β-catenin (Ctnnb1) and Cyclin D1 (Ccd1) (Fig. 4F) is linked with suture closure.31 Immunohistochemistry revealed elevated expression of cell proliferation marker protein in the osteogenic front of calvaria of MS-275-treated newborn mice (Fig. 4G). Moreover, administration of a low dose of MS-275 stimulated cell growth of primary osteoblasts (Fig. 4H). These observations corroborate our speculation that MS-275 promotes cell proliferation, thereby accelerating the process of suture closure. Collectively, these data indicate that Runx2 stimulating effect of MS-275 can increase osteoprogenitor cell number in suture space by stimulating proliferation and stimulate further differentiation of these cells in Runx2/−/− mouse calvarial cells and tissue.

**Table 1. Genes Differentially Regulated in Calvarial Bones From WT and Runx2/−/− Mice**

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MS-275-mediated changes in expression of various biological processes (BP)-related genes. Log2 (fold change) values and p values for WT/MS-275 (MS) against WT/vehicle (veh); Runx2/−/− (Het)/vehicle toward WT/vehicle; Runx2/−/−/MS-275 to Runx2/−/−/vehicle; and Runx2/−/− (Het)/MS-275 against WT/vehicle. The 26 genes listed were selected as significantly (≥1.3-fold, p ≤ 0.05) differentially expressed genes (DEGs).

*p < 0.05; **p < 0.1; ***p < 0.001.

**Discussion**

Previously, it was suggested that a threshold level of RUNX2 is required for the normal skeletal development8 and that RUNX2 is deficient in human CCD patients and Runx2/−/− mice.30 In this study, we tested the hypothesis that restoration of Runx2 activity by MS-275 treatment could prevent development of CCD-like phenotypes in Runx2/−/− mice. Indeed, our results show
that fetal administration of MS-275 during early skeletal development rescued Runx2+/− mice from CCD-like phenotypes related to development of the cranial suture, mandible, and clavicle, although it is still partial (Fig. 1). Rescuing CCD-like phenotypes could be more effective when we adjust the initiation stage, dosage, and frequency of drug treatment with pharmacokinetic analysis. However, because we administered chemicals in utero, there was a limitation of using Runx2+/− mice for this study; we could not rule out the maternal effects on the embryos during pregnancy. To overcome this limitation, we showed double fluorescence labeling assay accompanied with organ culture system. In this study, we showed MS-275 exerted a much stronger effect on intramembranous rather than endochondral bone formation. In contrast to the prominent effect in flat bones, long bone development was neither significantly perturbed by Runx2 deficiency nor significantly improved by MS-275 (Supplemental Fig. S2). Clavicles develop by both endochondral (lateral part) and intramembranous (medial part) bone formation processes. In hypoplastic clavicles, the medial part is usually missing. We found that MS-275 treatment primarily restored the medial part of the clavicles in Runx2+/− mice, indicating that intramembranous bone formation is more sensitive to the restoration of Runx2 function by MS-275 treatment (Supplemental Fig. S1). Compared with other membranous bones like calvaria and mandible, MS-275 did not restore clavicle formation that markedly. This may be because clavicles are among the earliest bones to develop, whereas we initiated MS-275 administration at 14.5 dpc, potentially too late to recover clavicles. Thus, earlier administration might be preferable for this purpose. These results demonstrated that genetic insufficiency of a gene owing to a single-allele loss-of-function mutation can be rescued by increasing protein activity or stability with specific modulators. This approach could also be applied to the treatment of other single-gene genetic disorders.

Next, we addressed the mechanism underlying the effects of MS-275 on CCD-like phenotypes. First, we are suggesting MS-275 has a role in Runx2 posttranslational modification because it can acetylate Runx2, generating the functionally activated form. As expected, acetylated Runx2 was stabilized and spared from degradation, and both its abundance and transactivating activity increased (Fig. 2). Nonetheless, it remains possible that the level of Runx2 protein was indirectly elevated, resulting from an increase in Runx2 mRNA expression in response to MS-275 treatment, as shown in Fig. 2A. Therefore, to rule out this background, we carried out a tag-detection system in immunoblot after overexpression of MYC-tagged-Runx2 (Fig. 2D). Previous study also demonstrated by using overexpression of a tagged-Runx2 system that HDAC deacetylates Runx2 and HDIs increase its acetylation. These results indicate that MS-275 can enhance Runx2 protein activity in the absence of transcriptional upregulation of endogenous Runx2. Second, MS-275 can modify the epigenetic landscape. Specifically, the drug decreased the methylation level of the CpG island in the Runx2 promoter 2, thereby stimulating Runx2 expression. This observation is consistent with previous reports that MS-275 promotes both histone acetylation (by inhibiting HDAC) and CpG demethylation (by repressing DNA methyltransferase). In aspects of histone modification, our results of ChIP assay and immunoblot assay were also comparable with previous study, which demonstrated marked histone modification dynamics during osteoblast differentiation (Fig. 3B, Supplemental Fig. S4). In the process of osteogenesis, promoter region of Runx2 experienced elevated H3 and H4 acetylation and reduced H3K9, H3K27 methylation, whereas global level of those hallmarks were not changed. Collectively, these changes promote Runx2 transcription (Fig. 3A). Together with posttranslational modification of Runx2 protein, the positive effect on transcription may be sufficient to reach the threshold Runx2 level required to overcome genetic haploinsufficiency.

What, then, is the functional consequence of Runx2 activation in the early skeletogenesis? Our RNA-seq results indicated that MS-275 strongly stimulated the expression of osteoblast marker genes, as was well known from previous studies. In addition, we observed significant upregulation of proliferation-related genes and downregulation of apoptosis-related genes (Fig. 4, Table 1). Previously, we showed that many HDIs can stimulate Runx2 activity. These observations are correlated by the study of Schroeder and colleagues. They showed increased ALP activity in calvarial tissue-cultured media after MS-275 treatment and suggested MS-275 has a role in enhancing Runx2 transcriptional activity. In this study, we found that MS-275 has a clear therapeutic effect on CCD. This might be explained in any of several ways. First, the differences in the effects of MS-275 may be attributed to its distinct specificities on HDAC1 and 3. MS-275 preferentially inhibits HDAC1 and 3 with IC50 of 0.51 and 1.7 μM, correspondingly. Previous reports showed that HDAC1 binds to promoters of the bone marker genes osterix and osteocalcin to repress their expression. In addition, HDAC3, a well-known transcriptional co-repressor of Runx2, works as a negative regulator of lineage-committed osteoblasts by binding to NFATc1, Zfp521, TCF, and Runx2. Collectively, these facts could explain, at least in part, the successful therapeutic effect of the class I–specific inhibitor MS-275. Second, marked stimulating activity toward cell proliferation might cause distinct CCD recovery effects. MS-275 presented effective stimulation on expression of proliferation marker genes and proliferation process.

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![Fig. 5. Mechanism of Runx2 activation by MS-275. MS-275 regulates Runx2 expression via two mechanisms: 1) stabilization of Runx2 protein through posttranslational modification (PTM), which induces Runx2 acetylation and protects Runx2 from ubiquitination; 2) epigenetic activation of the Runx2 promoter region via alteration of the epigenetic landscape. MS-275 activates the Runx2 promoter by modifying chromatin structure from heterochromatin to euchromatin by increasing histone acetylation and decreasing CpG and histone methylation. HAT = histone acetyl transferase; Ac = acetylation.](image-url)
We Acknowledgments

Dhx36, (Fig. 10) also known for its critical roles in the process of cell proliferation. Therefore, we deduced that Dhx36-mediated signaling might be participating in highly triggered cell proliferative activity through MS-275 administration. Considering the significance of proliferation of osteoblast progenitor cells in the calvarial suture region during development, it could be the critical distinction of MS-275 treatment toward CCD.

Based on our findings and previous reports, we conclude that the combined functions of MS-275 are responsible for the effects: Runx2 activation through PTM and transcriptional activation of Runx2 via epigenetic regulation (Fig. 5). To reach comparable therapeutic effect with a single mechanism, just by PTM or by epigenetics, the therapeutic dosage might be increased higher to generate more side effects. The dosage of MS-275 in this study is much less than one tenth of other experiments and the litter number of MS-275-treated mice was comparable to that of untreated control. These results indicate a combination of both mechanisms synergized to enhance Runx2-specific activity of the agent with a lower dose. In addition, the enhanced Runx2 function by MS-275 stimulates osteoblast proliferation and differentiation processes. However, we must be cautious when interpreting the relationship between cell proliferation and MS-275 because it also has antiproliferative effects and apoptosis-inductive properties as an anti-cancer reagent. However, many studies show that the effect of HDIs including MS-275 on cell proliferation is concentration-dependent; in general, they promote proliferation at low dose but inhibit growth at high dose. Although we could not observe any skeletal malformation as a side effect of HDI administration, some kinds of HDIs—valproic acid and trichostatin A (TSA), for instance—were reported as teratogenic reagents for developing mouse skeletons, causing embryonic skeletal malformation including vertebral or rib fusions at high-dose administrations. Thus, more extensive toxicological study would be required to apply HDIs in the clinical field.

Because regulation of Runx2 activity is of tremendous interest from the standpoint of developing therapeutic agents against skeletal diseases, our findings have important medical implications. Notably, we demonstrated that MS-275 could compensate for genetic insufficiency of Runx2 in Runx2-/- mice. Therefore, the use of this drug represents a promising chemopreventive strategy for a genetic disease, CCD, especially if it can be diagnosed before birth. MS-275 also holds tremendous potential as a therapeutic agent for diseases involving the Runx2 gene, including many bone disorders. Thus, our results provide a novel experimental and theoretical basis for developing therapeutic agents against CCD and other skeletal diseases.

Disclosures

All authors state that they have no conflicts of interest.

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Authors’ roles: Study design: HB, KY, KC, and HR. Study conduct: HB and HR. Data collection: HB, BK, HS, and SC. Data analysis: HB, YC, RI, JL, and MS. Data interpretation: KC, SB, HK, KW, JB, YL, and HR. Drafting manuscript: HB, YL, and HR. HR takes responsibility for the integrity of the data analysis.

References


