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Epigenetic drugs as new therapy for tumor necrosis factor-α-compromised bone healing

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ABSTRACT

Impaired bone regeneration by excess inflammation leads to failure of bone healing. Current therapies display limited benefits making new treatments imperative. Our recent discoveries of the anti-inflammatory characteristics of bromodomain and extra terminal domain (BET) inhibitors, *N*-methylpyrrolidone (NMP) and *N*,*N*-Dimethylacetamide (DMA), implicate possible therapeutic use of epigenetic drugs in inflammation-impaired bone healing.

Here, we investigated the effects of NMP and DMA on osteogenesis *in vitro* and *ex vivo* under the influence of TNFα, a key cytokine responsible for impaired fracture healing. NMP and DMA pre-treatment recovered TNFα-inhibited expression of essential osteoblastic genes, Alp, Runx2, and Osterix as well as mineralization in multipotent stem cells, but not in pre-osteoblasts and calvarial osteoblasts. The mechanism of action involves the recovery of TNFα-suppressed BMP-induced Smad signaling and the reduction of TNFα-triggered ERK pathway. In addition, ERK inhibitor treatment diminished the effect of TNFα on osteogenesis, which reinforces the role of ERK pathway in the adverse effect of TNFα. Furthermore, endochondral ossification was analyzed in an *ex vivo* bone culture model. TNFα largely abrogated BMP-promoted growth of mineralized bone while pre-treatment of NMP and DMA prevented the deleterious effect of TNFα. Taken together, these data shed light on developing low- affinity epigenetic drugs as new therapies for inflammation-compromised bone healing.

1. Introduction

Bone fractures are the most common and costly traumatic injuries worldwide. In the US, there are approximately 15 million fracture cases annually with the healthcare expenditures of more than 60 billion dollars. Bone fractures thus pose a substantial socioeconomic burden [1] on top of the dramatic reduction in life quality of the patients.

A bone fracture occurs when the force applied on bone exceeds its strength, leading to a break, subsequently reducing the mechanical stability of the bone. Although the skeletal system features extraordinary regenerative capacity to repair fractures, about 10% of fractures fail to heal normally [2]. Failure of the fracture healing is due to impaired bone regeneration process that causes an increased risk of developing deleterious complications, such as non-unions [3].

Bone regeneration during fracture healing is a well-orchestrated physiological process, and many factors can interfere with it. However, excess inflammation triggered by additional injuries or comorbid diseases is described as the most common cause [4]. Unlike controlled inflammation which is required for normal fracture healing, excess inflammatory responses create a systemic increased expression of several pro-inflammatory cytokines [3] among which tumor necrosis factor-α (TNFα) is a critical cytokine associated with impaired new bone formation [5]. Several studies have shown that high level of TNFα inhibits the expression of various osteogenic genes, thus blocking osteogenic differentiation of mesenchymal stem cells (MSCs) and the maturation of osteoprogenitors [6–14]. Hence, anti-TNFα therapeutics have been developed to treat inflammation-impaired bone regeneration. However, current anti-TNFα agents show unsatisfactory clinical benefits so far [15]. Thus, effective therapies to fine-tune the pathological action of TNFα are still lacking. For that, an emerging class of therapeutics, called epigenetic drugs, offers alternative treatment options to explore.

Epigenetics refers to the heritable modifications that control gene expression without altering the genetic sequence in the genome [16].

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Common epigenetic modifications involve DNA methylation, histone modification (*i.e.*, acetylation and methylation), and non-coding RNA (*i.e.*, lncRNA). Bromodomains are "readers" that bind acetylated lysines in histone tails, and their most important function is the regulation of gene transcription by the recruitment of different molecular partners. Thus, proteins containing bromodomains are considered as epigenetic regulators. Since the activity of epigenetic factors is likely to be chemically modulated, they are ideal targets for controlling gene expression and for modulating their activity. These characteristics offer considerable potential for the development of new drugs [17]. Recent report of the clinical applicability of a BET inhibitor, (+)-JQ1 in treating osteoporosis revealed that BET inhibitors might be promising therapeutics for inflammation-related bone disorders. [18].

Recently, two FDA-approved drug excipients, *N*-methylpyrrolidone (NMP) and *N*,*N*-Dimethylacetamide (DMA) were discovered to act as BET bromodomain inhibitors [19–21]. Notably, studies have demonstrated that both NMP and DMA reduce inflammation and favorably influence bone remodeling *in vivo* by suppressing osteoclastogenesis and promoting the anabolic effect of BMP2 [19,20,22,23]. Taken together, these results suggest that they are candidates for developing effective treatments for inflammation-impaired bone healing. However, the data regarding the effects of NMP and MDA on osteoblast differentiation under excess inflammation is lacking.

To that end, we investigated the ability of NMP and DMA to counteract the deleterious effect of TNFα on osteogenesis *in vitro* and on metatarsal bone growth *ex vivo*, a widely used model to study new bone formation.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's modified eagle medium (DMEM) with GlutaMAX supplement, minimum essential medium eagle-alpha (MEM-α), fetal bovine serum (FBS), L-glutamine, penicillin, amphotericin B, streptomycin, protease and phosphatase inhibitors cocktail, NE-PER nuclear/cytoplasmic extraction reagents, and collagenase were obtained from Thermo Fisher Scientific (Waltham, Ma, USA). Primers for RT-PCR (QuantiTect primer assay) and RNA extraction kit (RNeasy kit) were purchased from Qiagen (Hilden, Germany). iScript reverse transcription supermix, SsoAdvanced universal SYBR green supermix, and Bradford protein assay reagent were obtained from Bio-Rad Laboratories (Hercules, CA, USA) The BCA kit for protein determination was from Pierce. Anti-RUNX2 (D1L7F), anti-pSmad1/5/9 (D5B10), anti-p-NK-κB p65 (93H1), anti-NK-κB p65 (D14E12), anti-pp44/42 MAPK (D13.14.4E), anti-p44/42 MAPK (137F5), anti-pp38 (D3F9), anti-p38 MAPK (D13E1), anti-pSAPK/JNK (81E11), anti-GAPDH (D16H11), anti-TBP (D5C9H), and anti-rabbit-IgG HRP-linked antibodies were purchased from Cell Signaling Technology (CST, Inc., USA). Anti-Sp7 (ab22552), anti-Smad1/5/9 (ab66737), and anti-SAPK/JNK (ab208035) were from Abcam (Cambridge, UK). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell cultures

Both C2C12 and MC3T3-E1 cell line was purchased from American Type Culture Collection (ATCC). C2C12 and MC3T3-E1 were cultured in DMEM + GlutaMAX-I and MEM-α medium without ascorbic acid, respectively. All culture media were supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 units/ml streptomycin). Cells were incubated at 37 °C with 5% CO_2 in humidified air. The osteogenic medium for MC3T3-E1 consisted of MEM-α supplemented with 50 μg/ml L-ascorbic acid and 10 mM β-glycerol phosphate. Bone-marrow derived stem cells (BM-MSCs) were isolated from long bone of 6-week-old Sprague Dawley female rats as described previously [24], and were then maintained in DMEM + GlutaMAX-I medium. Rat calverial osteoblasts (rOBs) were isolated as described in [25] and further cultured in DMEM. For osteogenesis, medium was supplemented with 50 μg/ml ascorbic acid, 2 mM β-glycerol phosphate, and 10 nM dexamethasone.

2.3. Alkaline phosphatase (ALP) activity assay and staining

The protocol was previously described in [21]. In short, cells were seeded at a density of 5×10^4 cells/cm^2 in 6- or 24-well plates (n = 3 per group) for 16–24 h before incubation with indicated molecules. After at least six days of incubation, cells were washed with PBS and then scraped in 0.56 M 2-amino-2-methyl-1-propanol. After homogenization and subsequent centrifugation, the supernatants were collected and tested for ALP activity using *p*-nitrophenyl phosphate as a substrate. The protein content of the lysates was determined using Bradford protein assay reagent for normalization purpose. To examine alkaline phosphatase activity histochemically, cells were fixed for 10 min with 10% Formalin (1 ml per well in 6-well plates) at room temperature. After washing with PBS, cells were stained as in [19]. Photos of the stained cells were taken using CCD camera.

2.4. Quantitative real-time RT-PCR

RNA was extracted using the RNeasy mini kit (Qiagen) and then reverse transcribed into cDNA using iScript reverse transcription supermix for qRT-PCR (Bio-Rad). The resulting cDNA was subjected to real-time PCR with gene-specific primers (QuantiTect primer assay, Qiagen, see Table 1 for details) using SsoAdvanced universal SYBR green supermix and the CFX Connect real-time system (Both were from Bio-Rad).

2.5. Western blot analysis

Total cell lysates were extracted using RIPA buffer supplemented with protease and phosphatase inhibitors cocktail and subjected to analysis as previously described [19]. For specific experiments, nuclear cell extracts were isolated using NE-PER nuclear and cytoplasmic extraction reagents. An equal amount of proteins was separated on a 4–20% precast polyacrylamide gel and transferred to a PVDF membrane using the precast Trans-Blot turbo stack (All were from Bio-Rad). Proteins were detected by appropriate primary antibodies and HRP-linked secondary antibody. The signals of proteins were detected using Clarity Western ECL substrate and ChemiDoc MP imaging system (Both were from Bio-Rad).

2.6. Alizarin red staining

Cell were washed with PBS and fixed using 70% ethanol for 1 h. Next, cells were stained with Alizarin red solution as described in [21]. The stained cells were then photographed.

2.7. Immunofluorescence (IF) staining

Cells were seeded on 12-well chamber slides (Ibidi, Planegg, Germany) 24 h before stimulation. Cells were then stimulated with indicated molecules for specified time and fixed with 4% formaldehyde.

Table 1
Primers for qPCR.

Gene	Species	Company	Catalog nr.
Sp7_Osterix	Mouse	Qiagen	QT00293181
Runx2	Mouse	Qiagen	QT00102193
Alp	Mouse	Qiagen	QT00157717
Gapdh	Mouse	Qiagen	QT01658692

Specimens were rinsed with PBS, followed by blocking with blocking buffer (1× PBS/5% normal serum/0.3% Triton™ X-100) for 60 min. Samples were incubated with anti-NK-κB p65 antibody (1:400) diluted in antibody dilution buffer (1× PBS/1% BSA/0.3% Triton™ X-100) for 24 h at 4 °C, followed by incubation with anti-rabbit IgG (H + L), F(ab′) 2 Fragment (Alexa Fluor® 488 Conjugate) for 1–2 h. Afterward, the samples were incubated with DyLight™ 554 Phalloidin for 20 min and then with Prolong® Gold Antifade Reagent with DAPI (CST). Lastly, slides were cured in the dark for overnight at room temperature before imaging with ZOE™ Fluorescent cell imager (Bio-Rad).

2.8. Metatarsal bone culture

The model was adopted from [26]. Briefly, metatarsal bones from hind limbs of Sprague Dawley pups (post-natal day one, PN1) from the same litter were isolated. All the metatarsals, except the first and fifth ones, were carefully dissected and transferred into the wells on 24-well plates with 200 μl/well of MEM-α medium without ribonucleosides and supplemented with 0.2% BSA (Sigma-Aldrich), 5 μg/ml L-ascorbic acid 2 phosphate, 1 mM β-glycerol phosphate, 100 units/ml Penicillin and Streptomycin, and 1.25 μg/ml fungizone. Metatarsals were then stimulated with indicated molecules for 14 days. Medium was changed every 3 days throughout the culture period. To assess endochondral ossification of metatarsals, images were taken with CCD camera on the first day and the last day of the culture period, followed by the calculation of changes in mineralized length using ImageJ Software (NIH, USA).

2.9. Statistical analysis

All statistical analyses were performed with GraphPad Prism 7. Results from *in vitro* or *ex vivo* studies were expressed as the mean ± SD and were compared by Student's *t*-test or ANOVA, respectively. All results were considered significantly different for $p < 0.05$.

Key resources table

Resource	Source	Identifier
Antibodies		
Anti-pSmad1/5/(D5B10)	Cell Signaling Technology	#13820
Anti-GAPDH	Cell Signaling Technology	#5174
Anti-p44/42 MAPK (137F5)	Cell Signaling Technology	#4695
Anti-pNFkB 65	Cell Signaling Technology	#3033
Anti-pp38 (D3F9)	Cell Signaling Technology	#4511
Anti-pSAPK	Cell Signaling Technology	#4668
Anti-rabbit-IgG HRP-linked	Cell Signaling Technology	#7074
Anti-RUNX2	Cell Signaling Technology	#12556
Anti-SAPK/JNK	Abcam	ab208035
Anti-Smad1/5/9	Abcam	ab66737
ANTI-Sp7	Abcam	ab22552
Anti-TBP	Cell Signaling Technology	#44059
CellLine		
MC3T3-E1	ATCC	CRL-2595
Chemical		
2-Amino-2-methyl-1-propanol	Sigma	
Alizarin red	Sigma	
Amphotericin B	Thermo Fisher Scientific	
Dexamethasone	Sigma	
Formaldehyde	Sigma	
Formalin	Sigma	
Fungizone	Thermo Fisher Scientific	
GlutaMAX-I	Thermo Fisher Scientific	
L-glutamine	Thermo Fisher Scientific	
L-Ascorbic acid 2 phosphate	Sigma	
Penicillin	Thermo Fisher Scientific	
p-Nitrophenyl phosphate	Sigma	
Prolong® Gold Antifade Reagent with DAPI	Cell Signaling Technology	#8961
β-Glycerol phosphate	Sigma	
Streptomycin	Thermo Fisher Scientific	
X-100	Sigma	
ProteinPeptide		
CFX	Bio-rad	
Collagenase	Thermo Fisher Scientific	

3. Results

3.1. Effects of NMP and DMA on TNFα-inhibited osteogenesis

To investigate the therapeutic effects of NMP and DMA against inflammation-repressed osteogenesis, we first examined their abilities to restore the expression of the early osteogenic marker, alkaline phosphatase (ALP), in C2C12 in the presence of TNFα. As mentioned in the introduction, TNFα has been linked to direct suppression of osteogenic differentiation and therefore used to mimic excess inflammation in our study. In C2C12 cells, NMP and DMA pretreatment significantly reversed the inhibitory effect of TNFα on Alp mRNA expression (Fig. 1A). Furthermore, staining and enzymatic activity of ALP were fully restored from TNFα suppression with the presence of NMP or DMA (Fig. 1B). In contrast, neither NMP nor DMA recovered TNFα-suppressed ALP activities (Fig. 1C) and mineralization (Supplemental Fig. 1A) in pre-osteoblastic MC3T3-E1 cells and primary rat calvarial osteoblasts (Fig. 1D). These results suggest that NMP and DMA are effective on TNFα-inhibited osteogenesis in cells before committing to the osteogenic lineage. Indeed, the data in C2C12 cells at diverse differentiation stages confirm that the counteractive effect of NMP against TNFα is largely diminished in cells that were already differentiated by BMP2 (Supplemental Fig. 1B and C). This observation supports the notion that cells before lineage specification are more sensitive to the pre-treatment of NMP and DMA.

Next, we assessed the effects of NMP and DMA on the expressions of both early and late osteoblast-specific transcription factors, Runx2 and Osx (Osterix). Both transcription factors are essential trans-activators, which drive various vital osteogenic-specific genes during osteogenesis and osteoblast maturation [27,28]. As shown in Fig. 2A, NMP and DMA pretreatment reversed TNFα-reduced Runx2 mRNA level and restored RUNX2 protein to a similar degree as in the rhBMP2 only-treated group (Fig. 2C). Osx, on the other hand, was partially restored by the pretreatment of NMP or DMA at both mRNA (Fig. 2B) and protein levels (Fig. 2D). Thus, NMP and DMA rescued the TNFα-induced impairments of osteogenesis by restoring the expression of Runx2 and Osx.

Since NMP and DMA are bromodomain inhibitors [19,20], we assumed that other bromodomain inhibitors might possess the same therapeutic characteristics. To that end, we decided to test another well-studied bromodomain inhibitor, JQ1. However, the data clearly shows that neither 0.1 μM nor 1 μM of JQ1 countered TNFα-suppression on osteogenesis (Supplemental Fig. 2). Taken together, we concluded that the therapeutic effects of bromodomain inhibitors in the context of TNFα suppression are NMP- and DMA-specific, and unrelated to their bromodomain binding affinity.

3.2. Effects of NMP and DMA on TNFα-impaired osteogenesis in primary mesenchymal stem cells

Next, we intended to study the therapeutic effects of NMP and DMA on rat primary bone marrow-derived mesenchymal stem cells (BM-

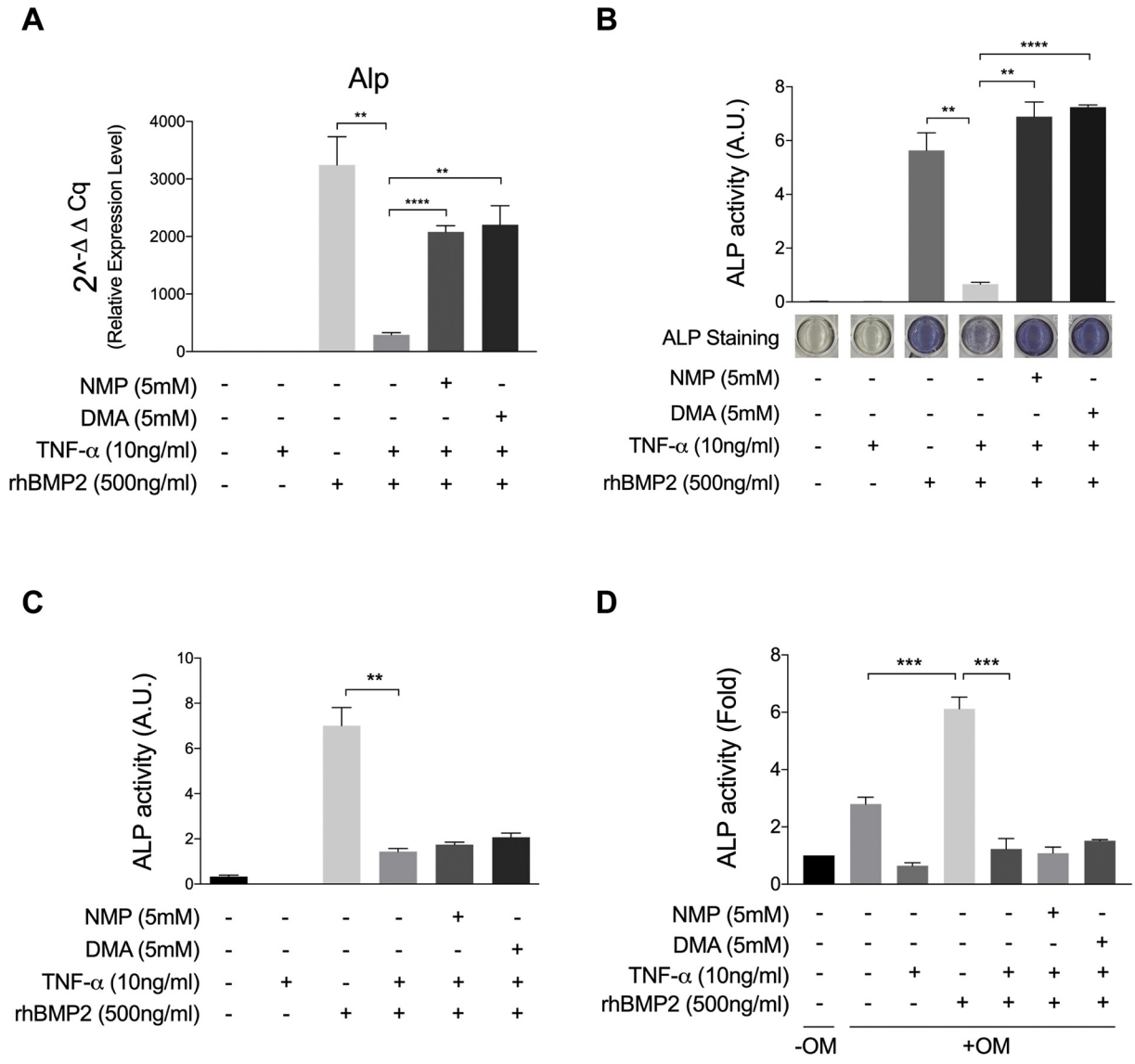

Fig. 1. NMP and DMA restored TNFα-inhibited osteogenesis in multipotent stem cells (C2C12), but not in pre-osteoblasts (MC3T3-E1) or rat primary calvarial osteoblasts (rOBs). Cells were pre-treated with NMP or DMA, followed by stimulation of indicated concentration of TNF-α and rhBMP2. (A) mRNA expression levels of early marker, Alp, were measured by qRT-PCR and (B) ALP activity was measured by ALP enzymatic activity assay as well as ALP staining, respectively, after at least 6 days in C2C12. (C) ALP activity was measured after 7 days using ALP enzymatic activity assay in MC3T3-E1. (D) ALP activity was measured after 7 days using ALP enzymatic activity assay in rOBs. OM: Osteogenic medium. Error bar indicates S.D. from triplicate samples. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

MSCs). Evidence has suggested that BM-MSCs are multipotent stem cells that differentiate into osteoblasts under the appropriate environmental cues [29]. Importantly, they are the primary source of replenishing osteo-progenitors during new bone formation needed to repair injured bone *in vivo* [29]. Hence, the study on MSCs could provide additional information to better predict the efficacy of NMP and DMA *in vivo*. As shown in Fig. 3A, NMP and DMA could protect MSCs against TNFα impaired osteogenesis based on the partial restoration of ALP activity. Besides, the data suggest that the effects of NMP and DMA are BMP2-dependent. Consistent with our finding in cell lines, studies demonstrate that NMP and DMA could rescue TNFα-suppressed mineralization only when the MSCs were at the early stage of osteogenesis (Fig. 3B), possibly during lineage commitment. Supporting evidence is shown in Supplemental Fig. 3 that after passing the early differentiation stage, the beneficial effects of pre-treating NMP and DMA were diminished whereas the deleterious effect of TNFα persisted.

3.3. Mode of action of NMP and DMA

To dissect the mechanisms by which NMP and DMA curtail the inhibitory effect of TNFα on osteogenesis, we examined several signaling pathways associated with BMP2 and TNFα during osteoblast differentiation. Firstly, key signaling driving osteogenesis [30], Smad1/5/9, was investigated (Fig. 4A). The analysis of the phosphorylation level of Smad1/5/9 shows that TNFα significantly reduced the amount of BMP2-phosphorylated Smad1/5/9, whereas the addition of NMP and DMA could fully restore the level of Smad phosphorylation. Therefore, NMP and DMA counteract the effect of TNFα by recovering TNFα-suppressed activation of Smad pathway.

TNFα mainly activates NF-κB pathway to facilitate bone resorption *via* promoting osteoclasts differentiation and to reduce new bone formation *via* suppressing osteoblast differentiation. Eventually, the increasing osteoclast-to-osteoblast ratio results in compromised bone regeneration [14,31,32]. Previous studies demonstrated that both NMP and DMA inhibit LPS-induced inflammatory responses in macrophages

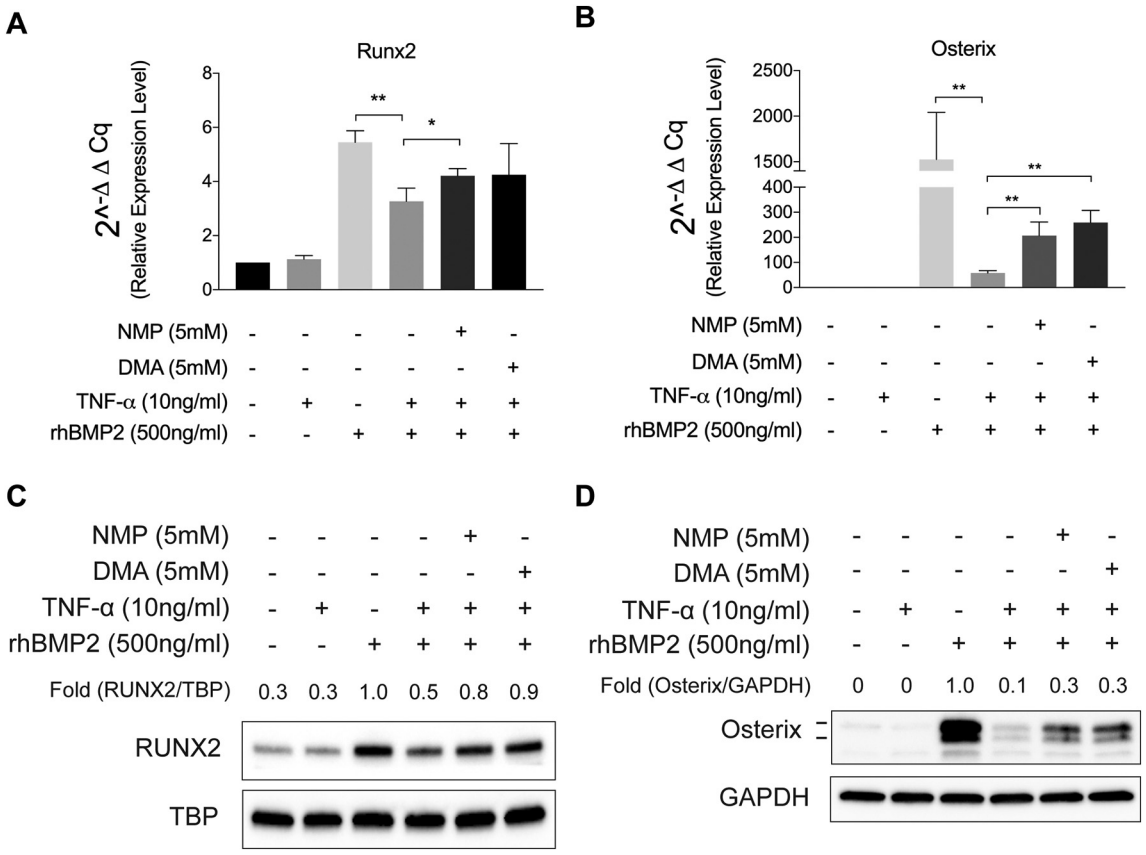

Fig. 2. NMP and DMA recovered TNFα-suppressed expression of crucial osteoblast-specific transcription factors, Runx2 and Osterix, in C2C12. Cells were pre-treated with NMP or DMA, followed by stimulation of indicated concentration of TNF-α and rhBMP2. (A and C) Runx2 mRNA and nuclear protein levels were assessed after 72 h of stimulation by qRT-PCR and western blot, respectively. TBP (TATA-binding protein) served as a nuclear marker. (B and D) Osterix mRNA and protein levels were analyzed after 120 h of stimulation. TBP and GAPDH were loading control. Error bar indicates S.D. from triplicate samples. *: $p < 0.05$; **: $p < 0.01$.

by partially suppressing NF-κB pathway [19,23]. Therefore, we investigated the effects of NMP and DMA on TNFα-activated NF-κB pathway in C2C12. The analysis on phosphorylated p65, a transcription regulator tightly linked to the activation of NF-κB pathway, demonstrates that TNFα triggered the phosphorylation of p65. However, pre-treatment with NMP or DMA did not interfere with TNFα-induced phosphorylation of p65 (Fig. 4B). In accordance with this result, the nuclear translocation of p65 also shows that NMP and DMA did not affect TNFα-induced p65 nuclear translocation (Supplemental Fig. 4). The data suggest that the effects of NMP and DMA on TNFα suppression in multipotent cells is NF-κB-independent.

Next, we decided to examine mitogen-activated protein kinases (MAPKs) pathways, including ERK1/2, JNK1/2, and p38, which are known to modulate BMP2-induced osteogenesis [33–35]. Furthermore, MAPKs pathways have been linked to the inhibitory effect of TNFα on osteoblastic differentiation [8]. Here, we revealed that TNFα activated ERK pathway during BMP2-induced osteogenesis, particularly the phosphorylation of ERK2. NMP and DMA pretreatment suppressed the TNFα-triggered phosphorylation to its basal level (Fig. 5A). To confirm our finding, we used ERK inhibitor, U0126, to mimic the effects of NMP and DMA *via* direct suppression of TNFα-induced ERK activation. Consistent with the result shown in Fig. 5A, we found that pre-treatment with ERK inhibitor dose-dependently recovered TNFα-reduced ALP activities (Fig. 5B). This data indicate that TNFα-activated ERK pathway directly correlates to the suppressive effects on osteogenesis. In contrast, there were no clear differences in the phosphorylation levels for JNK (Supplemental Fig. 5A) and p38 (Supplemental Fig. 5B) between rhBMP2 + TNFα group and NMP or DMA pre-treated group. Hence, the data suggest that TNFα-inhibited osteogenesis is ERK-dependent.

3.4. Effects of NMP and DMA on TNFα-compromised new bone formation ex vivo

To validate the therapeutic effects of NMP and DMA in a system under physiological-like condition, we decided to test their efficacy in an *ex vivo* metatarsal bone culture (Fig. 6A). This model has been widely used to study the effects of bioactive molecules on linear bone growth and endochondral ossification [26]. Fig. 6B demonstrates that TNFα alone led to de-mineralization of metatarsal bone while rhBMP2 largely facilitated endochondral ossification, as evidenced by a significant increase in the mineralized length. Noticeably, the addition of TNFα in rhBMP2-treated bones resulted in complete suppression of endochondral ossification, while pre-treatment of NMP and DMA recovered the mineralization again. This result is consistent with our *in vitro* findings and provides compelling evidence indicating the therapeutic effects of NMP and DMA on the level of an *ex vivo* model system.

4. Discussion

Appropriate bone healing after fractures poses a great challenge in clinical practice. Despite the advances in fracture treatments, some issues that could interfere with the healing process remain unsolved, particularly excess inflammation. Excess inflammation caused by polytrauma or extensive tissue damages together with fractures have been shown to impair bone regeneration severely [4]. Current treatments simply antagonizing critical pro-inflammatory cytokines, such as TNFα [15], were reported to show minor efficacy. However, recent discoveries of epigenetic drugs on the treatment of inflammation-associated bone disorders mark the dawn of the development of new

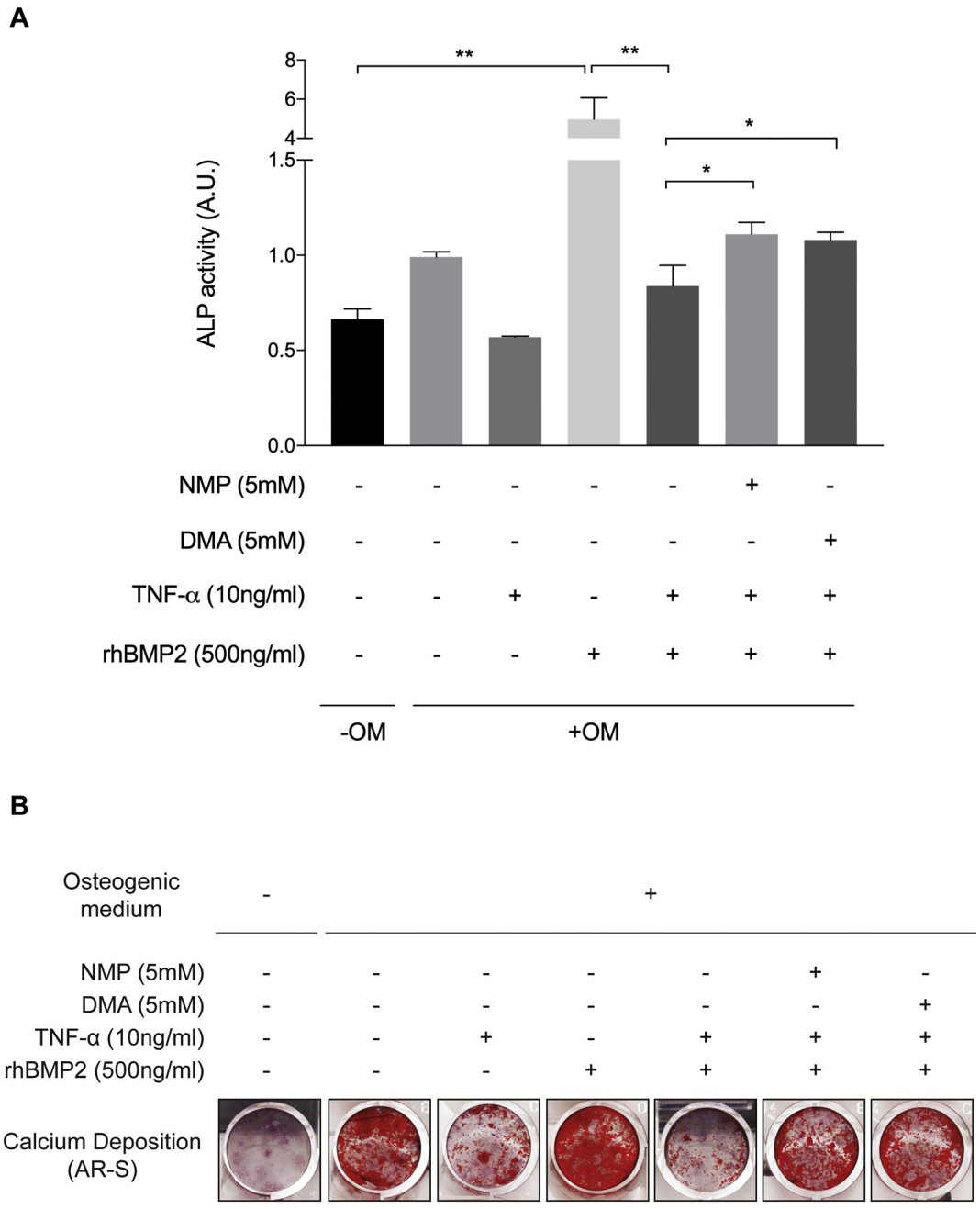

Fig. 3. Pre-treatment of NMP/DMA during lineage commitment could protect BM-MSCs against TNFα-suppressed osteogenesis. BM-MSCs were isolated from the femurs of male wild-type Sprague Dawley (SD) rats. (A) Cells were pre-treated with NMP or DMA, followed by stimulation of indicated concentration of TNF-α and rhBMP2 for 7 days. ALP activity was then measured using ALP enzymatic activity assay. (B) Cells were pre-treated with NMP or DMA, followed by stimulation of indicated concentration of TNF-α and rhBMP2 for the first 3 days, and then continued to differentiate with osteogenic supplements for 18 days. Culture medium was renewed every 3 days. After 21 days, calcium deposition was assessed *via* Alizarin red staining. Error bar indicates S.D. from triplicate samples. *: $p < 0.05$; **: $p < 0.01$.

therapy [18].

Recently, we showed that two FDA-approved small molecules, *N*-methylpyrrolidone (NMP) and *N*,*N*-Dimethylacetamide (DMA), are low-affinity, broadband BET bromodomain inhibitors [19,36]. Both NMP and DMA can inhibit inflammatory responses and osteoclast differentiation to attenuate bone resorption [19,22,23]. However, little is known about the effects of these BET inhibitors on osteoblast differentiation under inflammation. Here in this study, we found that NMP and DMA rescued the expression of Alp, Runx2, Osx at both mRNA and protein levels, thereby countering TNFα inhibition on osteogenesis in multipotent stem cells. Consistent with the data from cell lines, ALP activity and mineralization of MSCs were recovered by pre-treatment of NMP and DMA.

In contrast, pre-osteoblasts and primary osteoblasts did not respond to the pre-treatment of the inhibitors. These results suggest that the beneficial effects of NMP and DMA against TNFα are sensitive to the differentiation stages of cells. Thus, the time of the administration of NMP and DMA is critical during bone healing. The initial inflammatory phase during bone healing occurs from day 0 to day 3 when immune cells remove necrotic tissues and trigger angiogenesis to initiate repair. Afterward, the recruitment of MSCs and osteo-progenitors follows [2]. Since NMP and DMA are efficacious on the cells before lineage

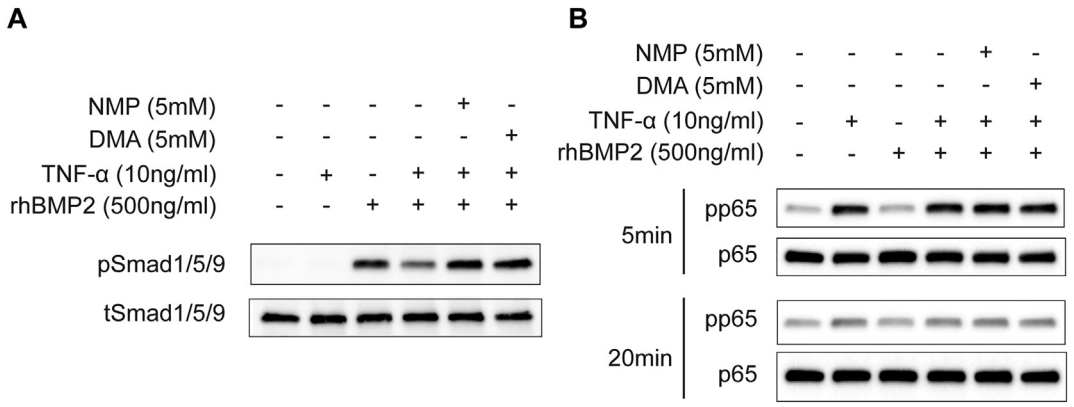

Fig. 4. NMP and DMA effects on Smad and NF-κB signaling pathways. (A) Cells were pre-treated with NMP or DMA, followed by stimulation of indicated concentration of TNF-α and rhBMP2 for 48 h. Total cell lysates were extracted and subjected to western blot analysis of the phosphorylation level of Smad1/5/9 complex. pSmad: phosphorylated Smad 1/5/9 complex; tSmad: total Smad 1/5/9/ complex served as a loading control. (B) Cells were starved with 1%FBS medium for 16 h, and then pre-treated with NMP or DMA, followed by stimulation of indicated concentration of TNF-α and rhBMP2. The amount of phosphorylated p65 and total p65 in cell lysates were measured at different time points (5 min and 20 min) by western blot analysis. The protein level of p65 served as loading control.

commitment, BET inhibitors should be applied locally at the healing site between day 2 and day 3. In this way, the controlled inflammation required for normal healing process will not be inhibited and the recruited multipotent stem cells will immediately encounter the BET inhibitors before osteogenic differentiation.

JQ1 is the most studied BET inhibitor that shows anti-inflammatory and anti-osteoclastogenic effects both *in vitro* and *in vivo* [37]. For the inflammation-compromised bone regeneration, JQ1 seems to be not suitable since it suppresses osteoblast differentiation and bone formation. The effect of JQ1 on osteoblast differentiation is probably due to its high affinity (nM range) compared to NMP and DMA (mM range). Moreover, JQ1 is not able to reverse the detrimental effect of TNFα on osteogenesis. Taken together, our data show that low-affinity broad-band bromodomain inhibitors, like NMP and DMA, which have additional activities like to enhance osteogenesis by increasing the phosphorylation activity of BMP-receptors for Smad [21] appear to have greater potential than JQ1 to improve bone regeneration in compromised situations.

Runx2 and Osx are transcription factors that drive the differentiation and maturation of osteoblasts. TNFα is described to down-regulate the expression of Runx2 and Osx, thus inhibiting osteoblast differentiation [7,8,12,38–40]. Our data demonstrate that NMP and DMA are able to recover TNFα-inhibited expression of Runx2 and Osx. For TNFα-inhibited Runx2 expression, our data shows that NMP and DMA fully recovered its protein level with a minor change in mRNA. One explanation for this discrepancy is that NMP and DMA might restore Runx2 expression by reducing its Smurf-mediated proteasome degradation. Both Smurf1 and Smurf2 are well-known E3 ubiquitin ligases responsible for ubiquitin/proteasome degradation of Runx2 during osteoblast differentiation [41,42]. To date, several pieces of evidence have suggested that TNFα inhibits osteogenesis *via* up-regulating Smurf1 and Smurf2, thereby promoting Smurf-mediated degradation of Runx2 [43,44]. Therefore, we reasoned that NMP and DMA are likely to suppress TNFα-driven Smurf1/2 expression, thus increasing the stability of Runx2 to protect MSCs from the deleterious effects of TNFα.

Smad pathway drives osteogenesis, and several studies indicate that TNFα reduces the phosphorylation of Smads to suppress osteoblast differentiation [8,11] whereas others suggest that the stimulation of TNFα does not change the phosphorylation of Smads in pre-osteoblasts [32]. These contradictory conclusions may be due to different cell types or differentiation stages. In our study, the result in multipotent stem cells supports the observation that TNFα inhibits osteogenesis *via* decreasing BMP2-driven Smad phosphorylation. Moreover, we demonstrate that pre-treatment of NMP and DMA could fully recover the TNFα-attenuated Smad signaling.

TNFα activates NF-κB signaling which has been widely studied as key signaling in osteoclasts. Emerging evidence further sheds light on its roles in regulating osteoblasts [31]. To date, it is known that TNFα

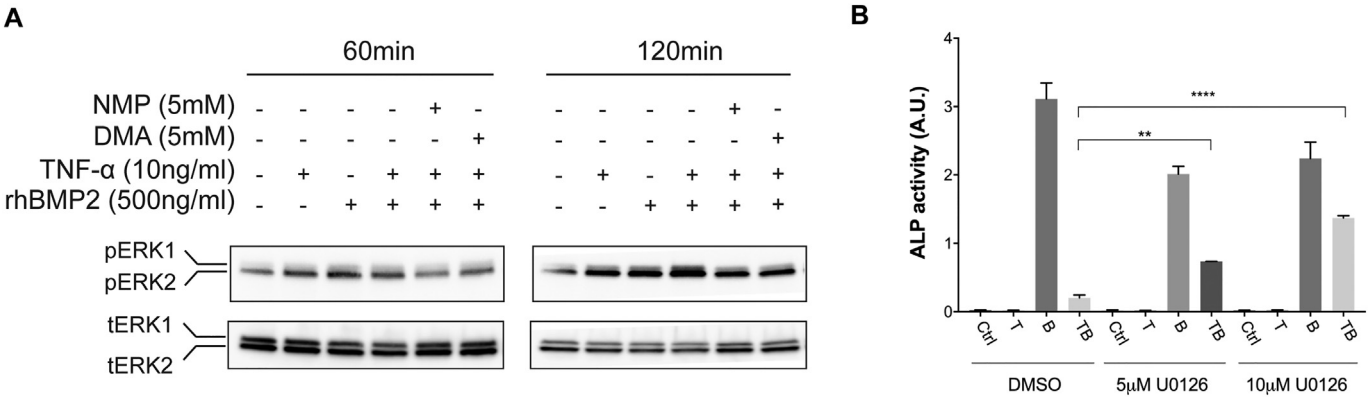

Fig. 5. NMP and DMA effects on ERK signaling pathway. (A) Cells were pre-treated with NMP or DMA and TNF-α, followed by stimulation of indicated concentration of rhBMP2 for 60 min. Cell lysates were isolated. Phosphorylation level of ERK1/2 was examined *via* western blot analysis. Total ERK served as a loading control. (B) Cells were first pretreated with control solvent (DMSO) or ERK inhibitor, U0126, respectively for 1 h. And then, cells were further treated with NMP or DMA, followed by stimulation of indicated concentration of TNF-α and rhBMP2 for 6 days. ALP activity was measured by ALP enzymatic activity assay. T: 10 ng/ml TNF-α; B: 500 ng/ml rhBMP2; TB: TNF-α + rhBMP2. Error bar indicates S.D. from triplicate samples. ***: $p < 0.001$; ****: $p < 0.0001$.

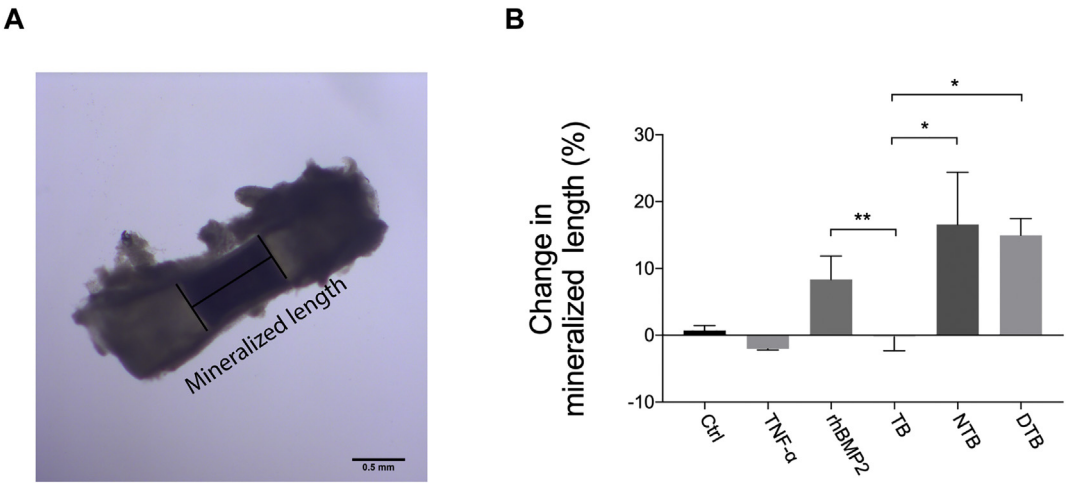

Fig. 6. Therapeutic effects of NMP and DMA on TNFα-compromised endochondral ossification of metatarsal bones. Metatarsal bones were extracted from P1 pups of Sprague Dawley (SD) rats and then cultured with NMP/DMA, TNFα, and rhBMP2 for 14 days. During culturing, images were taken on first and the last day for (A) the measurement of mineralized length in the end. (B) The changes in mineralized length were then calculated. TNF-α: 20 ng/ml; rhBMP2: 500 ng/ml; TB: TNF-α + rhBMP2; NTB: 20 mM NMP + TB; DTB: 20 mM DMA + TB. Error bar indicates S.D. from triplicate samples. *: $p < 0.05$; **: $p < 0.01$.

activates NF-κB signaling to suppress osteoblastogenesis, thus partially contributing to reduced bone formation [14,31,32,40,45–48]. Most of the studies on TNFα have focused on the components at the center of the classical NF-κB pathway, which is p65 (RelA). Upon TNFα stimulation, IKK (IκB kinase) triggers the phosphorylation and subsequent degradation of IκB (Inhibitor of κB). After the removal of IκB, p65 can be phosphorylated by kinases such as IKK, PKAc, MSK-1, and then the p65:p50 heterodimers are translocated into the nucleus to regulate transcription of NF-κB-target genes [45]. Accordingly, the phosphorylation and nuclear translocation of p65 are the hallmarks of NF-κB signaling activation.

In our study, NMP and DMA did not reduce TNFα-triggered nuclear translocation of p65 during osteoblastic differentiation. However, this result does not exclude the possibility that NMP and DMA might regulate the transcriptional activity of p65 at the epigenetic level. Considering that NMP and DMA are low-affinity inhibitors for BRD2,3,4 and BRDT [19,49], their abilities to interfere with activities of bromodomain (BD)-containing proteins which regulate the transactivation activity of p65 appear to be one potential explanation. From this perspective, Brd4 seems as an attractive target. Brd4 is associated with the regulation of the transcriptional activity of p65. The current working model illustrates that TNFα initiates NF-κB signaling, leading to p300-mediated K310 acetylation on p65. This acetylation further recruits Brd4, which serves as a coactivator of p65:p50 heterodimers and promotes the binding of CDK9 onto the promoters of NF-κB-target genes. Consequently, CDK9 phosphorylates C-terminal domain (CTD) of RNA Polymerase II to activate the NF-κB-dependent transcription of a subset of TNFα-responsive genes [50]. Taken together, in the context of TNFα-inhibited osteogenesis, NMP and DMA might block the activation of NF-κB-dependent genes, such as miR-150-3p [14] or Smurf1/2 [46], by interfering with the recruitment of Brd4 and CDK9. Therefore, NMP and DMA appear to facilitate the differentiation of MSCs to osteoblasts by blocking the NF-κB-mediated TNFα suppression of osteoblast differentiation.

Mitogen-Activating Protein Kinases (MAPKs), particularly ERK1/2, are signal transduction pathways working parallel with the NF-κB pathway to manifest the inhibitory effect of TNFα in MSCs and osteoblasts. Several previous findings have underlined the importance of ERK1/2 signaling in TNFα-mediated suppression on osteogenesis [8,11,40,48]. In line with earlier studies, our data indicate that TNFα increased the phosphorylation of ERK1/2, especially ERK2. Additionally, results from an inhibitor assay confirmed that ERK inhibitor successfully abrogated the repressive effect of TNFα on the expression of ALP. Our observation validates the notion that TNFα decreases osteoblast differentiation partially through activating ERK pathway. The novelty here is that we found that with the pre-treatment of NMP and DMA, TNFα-triggered phosphorylation of ERK2 was completely blocked. Altogether, our findings strongly suggest that NMP and DMA can counteract the inhibitory effect of TNFα on osteogenesis, at least in part, *via* attenuating TNFα-activated ERK signaling. In fact, ERK signaling is well known as primary mediator promoting proliferation [51]. In osteoblasts, studies have shown that the relative strength of ERK signaling could lead to different cell status. For instances, FGF activates strong ERK signaling but weak AKT signaling to inhibit differentiation whereas IGF-1 leads to weak ERK signaling yet robust AKT signaling to promote differentiation [52]. Given that differentiation and proliferation are mutually exclusive cellular events [53], it is likely that TNFα inhibits osteogenic differentiation *via* facilitating the proliferation of MSCs while the pre-treatment of NMP and DMA abrogate the proliferation-promoting effect of TNFα, thus allowing BMP2-driven osteogenesis to continue.

The metatarsal organ culture, well-established *ex vivo* system, is a highly physiological model for studying the effects of exogenous factors on bone growth and endochondral ossification as the growth rate of the bones mimic that observed in a physiological environment [26]. Here we demonstrated that the pre-treatment of NMP and DMA completely abrogated the inhibitory effect of TNFα on BMP2-promoted endochondral ossification. Notably, we observed that the treatment of TNFα alone reduced the length of the pre-existing mineralized matrix, probably through facilitating osteoclastogenesis and bone resorption [54]. This observation provides supporting evidence to the notion that excess TNFα could cause bone resorption resulting in compromised new bone formation during fracture healing.

5. Conclusion

In the present study, we revealed that NMP and DMA, two FDA-approved constituents of drug excipient recently characterized as BET inhibitors, could protect MSCs and osteoprogenitors from the deleterious effect of TNFα during osteogenesis. Mechanistically, NMP and DMA recover TNFα-inhibited expression of critical osteogenic genes, including Alp, Runx2, and Osterix. The therapeutic effects of these BET inhibitors are associated with their abilities to restore TNFα-reduced Smad signaling and to suppress TNFα-activating ERK pathway. Both activities appear to be independent of their bromodomain inhibition since high-affinity bromodomain inhibitors like JQ1 block osteogenesis.

Importantly, the efficacy of NMP and DMA was further validated in an *ex vivo* bone growth model where endochondral ossification was spared from TNFα inhibition with the treatment of these small chemicals. Overall, this study provides promising evidence of using NMP and DMA as an alternative treatment for inflammation-compromised bone healing in the future.

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