I-BET151 inhibits expression of RANKL, OPG, MMP3 and MMP9 in ankylosing spondylitis *in vivo* and *in vitro*

JIANPING FAN[1,2], JIAN ZHAO[1], JIE SHAO[1], XIANZHAO WEI[1], XIAODONG ZHU[1] and MING LI[1]

[1]Department of Orthopedics, Changhai Hospital, Second Military Medical University, Shanghai 200433; [2]Department of Orthopedics, Handan 285 Hospital, Handan, Hebei 056000, P.R. China

Received March 21, 2017; Accepted July 26, 2017

DOI: 10.3892/etm.2017.5032

Abstract. Ankylosing spondylitis (AS) is characterized by osteoclastogenesis and inflammatory bone resorption. The present study aimed to investigate the effect of bromodomain and extra-terminal domain (BET) protein inhibitor I-BET151 on AS process. A total of 38 AS Chinese patients were recruited and a further 38 sex- and age-matched healthy participants were selected as control. The Bath AS Function Index and Bath AS Disease Activity Index were assessed in AS patients and levels of erythrocyte sedimentation rate and C-reactive protein were measured in AS and healthy groups. Serum from AS patients was used to induce MG63 osteoblasts and BET inhibitor I-BET151 at concentrations of 50, 100 and 200 ng/ml used for treatment of the cells. A HLA-B27/β2m transgenic AS Lewis rat model was established and treated with 30 mg/kg I-BET151 for 5 weeks. Levels of receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), matrix metalloproteinase (MMP)3, and MMP9 were measured using ELISA *in vivo* and additionally detected with western blotting and polymerase chain reaction *in vitro*. The levels of RANKL, OPG, MMP3 and MMP9 were upregulated in AS serum, AS serum treated MG63 cells and HLA-B27/β2m transgenic AS rats. Conversely, levels of RANKL, OPG, MMP3 and MMP9 were significantly inhibited in cells or animals treated with I-BET151. Overall, the results of the present study demonstrated that BET inhibitor I-BET151 suppresses levels of RANKL, OPG, MMP3 and MMP9 in AS *in vivo* and *in vitro*. I-BET151 may exhibit the potential to be used as a therapeutic in the treatment of AS patients.

Correspondence to: Dr Ming Li, Department of Orthopedics, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, P.R. China
E-mail: liming_101@126.com

Key words: ankylosing spondylitis, I-BET151, inflammatory, matrix metalloproteinases

Introduction

Ankylosing spondylitis (AS) is a kind of chronic inflammatory disease which occurs in enthuses and spine with 2 basic characteristics, osteoporosis and ankylosis of axial joints (1). Studies show that elevated levels of inflammatory cytokines and matrix metalloproteinases (MMPs) are observed in AS patients, such as tumour necrosis factors, interleukins, MMP2, MMP3 and MMP8 (2-4). Recently, signal pathway of receptor activator of nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG) attracted scholars' attention. It was reported that increased RANKL could mediate osteoclastogenesis in AS patients or animal models (3). Moreover, bone resorption of skeleton could be protected by secreting OPG via osteoblasts. As reported, the balance of OPG and RANKL, as known as OPG/RANKL ratio, is important for maintaining normal bone metabolism. It is widely known that the OPG/RANKL ratio increases during the differentiation process of osteoblasts. In addition, upregulation of OPG/RANKL ratio has been well detected in AS patients (4-6).

Bromodomain and extra-terminal domain (BET) proteins is a 'histone reading protein' class and epigenetic-related proteins (3-5,7). It was reported that BET proteins played important roles in inflammatory bone resorption and osteoclastogenesis (3-5). In addition, BET bromodomain inhibitor could reduce osteosarcoma cell proliferation and osteoblastic differentiation (8-10). It was also reported that I-BET151, a BET bromodomain inhibitor, could regulate inflammatory factors in process of inflammatory diseases (7). However, there was no report focusing on the effects of BET inhibitors on the expression of OPG/RANKL system related factors in AS.

This study was designed to investigate the effect of I-BET151, a BET inhibitor, on the process of AS both *in vivo* and *in vitro* by AS cell model and animal model. The expression of RANKL, OPG, and MMPs including MMP3 and MMP9 in AS patients and animal serum as well as cells were detected.

Materials and methods

AS patients. A total of 38 AS Chinese patients were recruited from outpatient clinics at the Department of Orthopedics, Changhai Hospital, Second Military Medical University (Shanghai, China). All patients fulfilled the modified AS criteria of New York classification (4) and another 38 sex- and age-matched

healthy people were included In the present study as a healthy control. The Bath AS Function Index (BASFI, 0=none and 10=worst) and the Bath AS Disease Activity Index (BASDAI, 0=none and 10=worst) of AS patients were assessed (11,12). The participants demographic and disease characteristics were showed in Table I. Two inflammation markers including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels in serum from participants were detected. All the experiments performed In the present study followed the approval of the Ethics Ccommittee of Changhai Hospital and written informed consents were obtained from all participants.

Cells and treatment. A density of $2x10^5$ cells/ml of human MG63 osteoblasts (American Type Culture Collection, Rockville, MD, USA) were incubated in 6-well plates with DMEM (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 1% penicillin-streptomycin and 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C with 5% CO_2 for 24 h. Then cells were subjected into 250 μl of serum from AS patients in DMEM for another 48 h to induce AS cell model (4). For inhibition of RANKL/OPG system, MG63 cells were pretreated with 50, 100 and 200 ng/ml BET inhibitor I-BET151 (GlaxoSmithKline, Brentford, UK) for 2 h before AS serum addition (13,14). Each experiment was performed in triplicate.

AS animal model. HLA-B27/β2 m transgenic AS Lewis rat model was constructed as previously described (15,16). A total of 20 AS rats were constructed and all animals (including normal Lewis rats, n=10) were housed in standard conditions under a 12-h light/dark cycle with free access to food and water. For I-BET151 treatment, 20 transgenic rats were intraperitoneally administrated with 30 mg/kg of I-BET151 (n=10; GlaxoSmithKline) and equal volume normal saline (n=10) once per day for 5 weeks (10). At the end of 5 weeks, all animals were anesthetized and 0.5 ml of blood samples were collected before sacrifice. Animal procedures were performed according to Institutional Standards for Human Care and Use of Laboratory Animals.

ELISA assay. Blood samples collected from patients and animals were put into tubes and centrifuged at 1000 x g for 10 min and supernatants were collected for ELISA assay for RANKL, OPG, MMP3, and MMP9 (R&D Systems, Inc., Minneapolis, MN, USA). ELISA reader was used for absorption at 450 nm with batched controls.

Cellular RNA isolation and qPCR. Cellular RNA was extracted and the first-strand cDNA was synthesized for mRNA expression levels detection using ABI 7500 Real-Time PCR software (Applied Biosystems, Carlsbad, CA, USA) by a SYBR-Green PCR Master mix Kit (Applied Biosystems) according: denaturation at 94˚C for 4 min, 40 cycles of desaturating at 94˚C for 40 sec, annealing at 60˚C for 30 sec and elongation at 70˚C for 20 sec. Relative mRNA expression levels were calculated to reference GAPDH gene using the $2^{-\Delta\Delta Cq}$ method.

Western immunoblotting analysis. Cell lysates were prepared and separated using SDS-PAGE (10%). Then proteins were transferred to PVDF membrane. After being blocked with BSA, membranes were then incubated with primary antibodies anti-RANKL (1:1,000; BD Transduction Laboratories, San Jose, CA, USA), -OPG (1:1,000; Millipore Corp., Billerica, MA, USA), MMP3 (1:1,500; Millipore Corp.), MMP9 (1:1,000; BD Transduction Laboratories), GAPDH (1:2,000; BD Transduction Laboratories) at 4˚C overnight followed by incubation with secondary antibodies for 1 h and subjection to ECL reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) for visualization of western blots.

Table I. The participants demographic and disease characteristics of the study subjects.

Characteristics	AS patients	Healthy controls
Male/female, no. (%)	30/8 (78.9)	30/8 (78.9)
Age, years	31.4±6.0	32.3±5.6
Symptom duration, years	3.8±5.4	NA
HLA-B27 positive, no. (%)	33 (86.8)	NA
BASFI	3.0±3.1	NA
BASDAI	3.8±3.5	NA
CRP, mg/l	22.0±21.8	1.95±0.84
ESR, mm/h	23.9±18.6	4.63±1.25

AS, ankylosing spondylitis; HLA-B27, human leukocyte antigen B27; BASDAI, Bath AS Disease Activity Index; BASFI, Bath AS Functional Index; CRP, C-reactive protein (reference range, <6 mg/l); ESR, erythrocyte sedimentation rate (reference range, male <15 mm/h; female <20 mm/h); NA, not applicable.

Statistical analysis. Each experiment was performed in triplicate, and all data are showed as the mean ± standard deviation. Differences between groups and among groups were assessed using SPSS by analysis of Student's t-test or ANOVA, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of RANKL, OPG, MMP3, MMP9 in AS patients. The demographic and disease characteristics of the 76 participants are listed in Table I. Data showed that there was a higher AS accordance in man than that of women, and a 33 AS patients among the 38 patients were HLA-B27 positive (86.8%). Expression of RANKL, OPG, MMP3, and MMP9 in different groups was detected by ELISA and showed in Fig. 1. ELISA assay showed that the expression of RANKL, MMP3, and MMP9 was significantly upregulated in AS serum than those in healthy subjects, $P<0.05$. However, no significant difference was observed in OPG in AS patients compared with the healthy control, $P<0.05$.

I-BET151 inhibited AS serum induced expression of RANKL, OPG, MMP3, MMP9 in MG63 cell model. We treated MG63 cells with AS serum to induce the AS cell model *in vitro*. The expression of RANKL/OPG system factors RANKL, OPG, MMP3, and MMP9 mRNA and proteins were detected. The

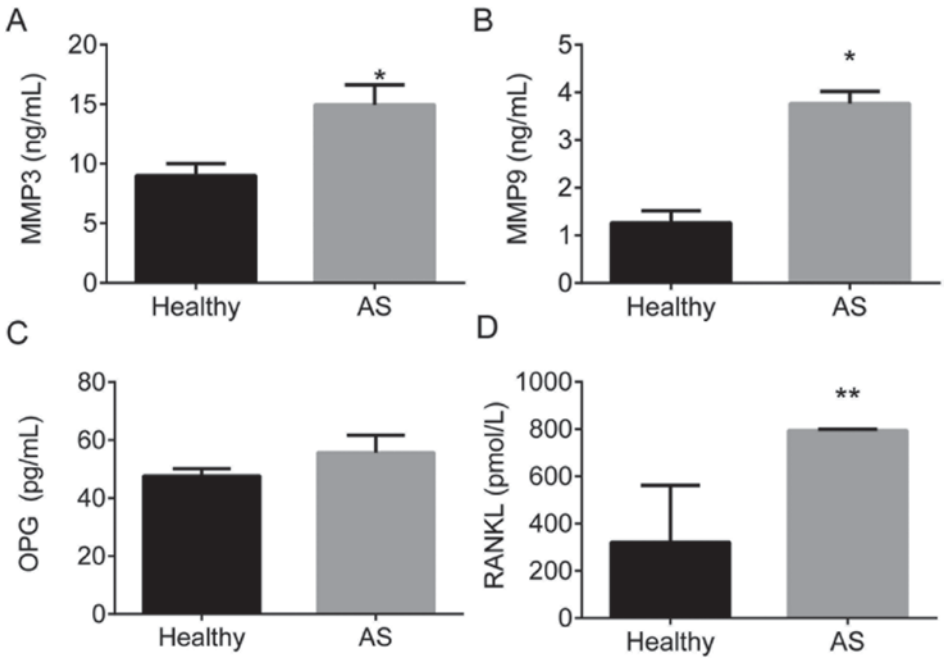

Figure 1. Expression of MMP3, MMP9, OPG and RANKL in AS patients. Expression of (A) MMP3, (B) MMP9, (C) OPG and (D) RANKL proteins were detected using ELISA assay. * and ** indicates difference at P<0.05 and P<0.01; AS vs. healthy controls. MMP, matrix metalloproteinase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand.

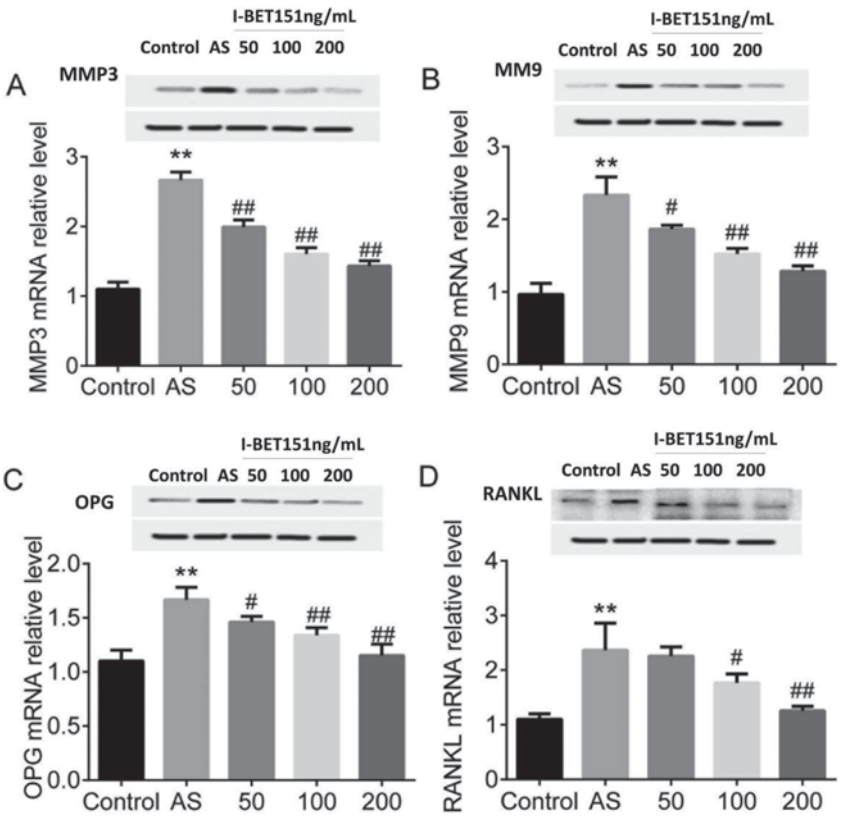

Figure 2. Expression of MMP3, MMP9, OPG and RANKL in AS MG63 cells. MG63 treated with AS serum were pretreated with 50, 100 and 200 ng/ml I-BET151. mRNA and protein expression of (A) MMP3, (B) MMP9, (C) OPG and (D) RANKL were detected using quantitative polymerase chain reaction and western blot assay. **, indicates difference at P<0.01, AS vs. control. # and ##, indicates difference at P<0.05 and P<0.01; I-BET151 vs. AS. AS, ankylosing spondylitis; MMP, matrix metalloproteinase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand.

results showed that both the mRNA and proteins of RANKL, OPG, MMP3, and MMP9 were significantly upregulated in AS serum induced MG63 cells compared with the control, P<0.01 (Fig. 2A-D). However, MG63 cells pretreated with I-BET151 showed significantly inhibitory effects on expression of RANKL, OPG, MMP3, and MMP9 in both mRNAs

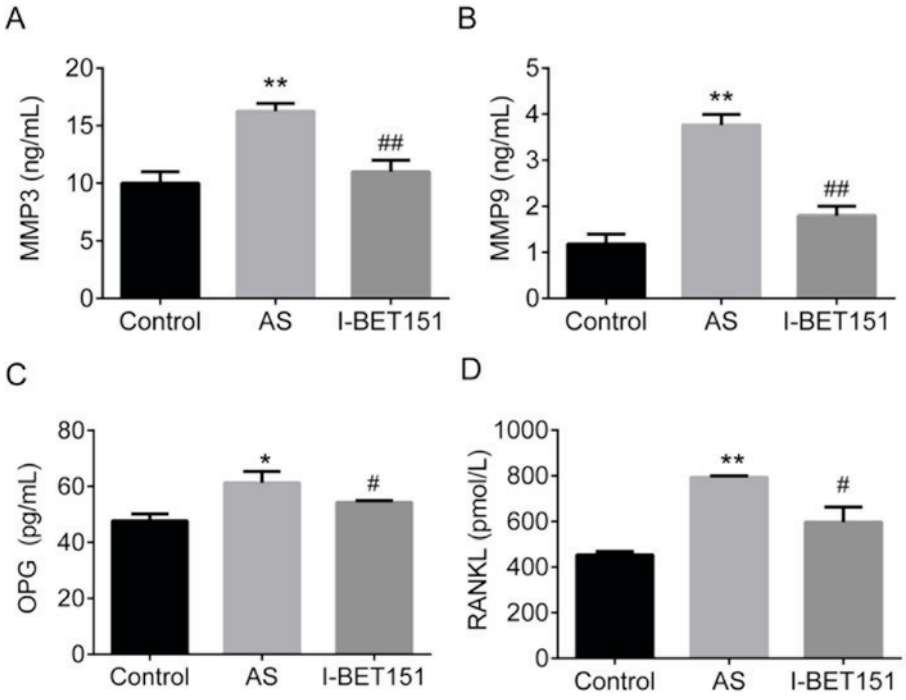

Figure 3. Expression of (A) MMP3, (B) MMP9, (C) OPG and (D) RANKL in AS rats. HLA-B27/β2 m transgenic Lewis rats were treated with 30 mg/kg of I-BET151 for 5 weeks, and serum were pretreated for ELISA. * and **, indicates difference at P<0.05 and P<0.01; AS vs. control. # and ##, indicates difference at P<0.05, and P<0.01; I-BET151 vs. AS. AS, ankylosing spondylitis; MMP, matrix metalloproteinase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand.

and proteins levels compared with MG63 AS model, with an dose-dependent manner. The expression of RANKL, OPG, MMP3, and MMP9 mRNAs and proteins showed the lowest expression levels in MG63 cells pretreated with 200 ng/ml I-BET151 than those of 50 and 100 ng/ml I-BET151, P<0.05. These results demonstrated that I-BET151 suppressed the expression of RANKL/OPG system.

I-BET151 suppressed expression of RANKL, OPG, MMP3, and MMP9 in AS rat model. We established the rat AS model using HLA-B27/β2 m transgenic Lewis rats. Levels of RANKL, OPG, MMP3, and MMP9 in rat serum were determines. The results showed that RANKL, OPG, MMP3, and MMP9 were upregulated in AS rats compared with the control rats, P<0.05 (Fig. 3). On the contrary, AS rats treated with 30 mg/kg of I-BET151 for 5 weeks showed significant inhibitory effects on levels of RANKL, OPG, MMP3, and MMP9 compared with the AS model, P<0.05. These data demonstrated that I-BET151 could inhibit AS induced expression of RANKL, OPG, MMP3, and MMP9 in AS rats.

Discussion

Previous reports have demonstrated the upregulation of MMPs, OPG, and RANKL in AS patients (2,3,6). However, to our best knowledge, there is no study focusing on effects of BET and its inhibitors in AS up to now. In the present study, we demonstrated the inhibitory effects of a BET inhibitor, I-BET151, on expression of MMPs, OPG, and RANKL in AS both *in vivo* and *in vitro*.

First we detected the expression of MMPs, OPG, and RANKL in AS patients as well as healthy control. Results showed that in AS patients all levels of above proteins increased. This result was in consistent with other studies. It was reported that RANKL and MMPs are inflammatory factors related to arthritis category (6). Association between RNKL expression and bone destruction was confirmed in inflammatory joints using animal model and the inflammatory cytokines of RANKL/OPG axis showed essential roles in AS process (4,17). In addition, MMPs upregulation, especially for MMP3, has been detected in AS serum and thus could be used as biomarkers for AS diagnosis (6,18).

Then we used AS serum to induce AS model *in vitro* and established AS rats model *in vivo*. Then we determined expression of MMPs, OPG, and RANKL both *in vitro* and *in vivo*. Results showed that all OPG, RANKL, MMP3 and MMP9, were significantly upregulated in AS serum treated MG63 cells as well as in HLA-B27/β2 m transgenic Lewis rats. However, when treated with I-BET-151, all these effects induced by AS were significantly inhibited.

The upregulation of RANKL and conflicting dysregulation of OPG in AS serum have been reported in numerous studies (6). Both the up-, down-, and no-regulation of OPG had been reported in AS studies. However, effect of BET inhibitor in AS is unknown.

The important role of BET bromodomain signaling in osteosarcoma is widely accepted, and the inhibition of it can suppresses growth of osteosarcoma tumour (8). Moreover, inhibition of BET protein suppresses osteoblastogenesis as well as osteoclast differentiation (19-21).

In the present study, we firstly demonstrated that I-BET151, a BET inhibitor, could suppress the upregulation of RANKL, OPG, MMP3, and MMP9 in AS serum treated MG63 cells and AS rats, suggesting the potential of using

I-BET151 as a therapeutic strategy for AS both *in vitro* and *in vivo*.

In conclusion, In summary, we demonstrated the upregulation of RANKL, OPG, MMP3, and MMP9 in AS *in vitro* and *in vivo*. The administration of BET inhibitor I-BET151 suppressed the expression of RANKL, OPG, MMP3, and MMP9 in AS in a dose-dependent manner. We concluded that I-BET151 might be used as a potential therapeutic strategy for AS patients after more future clinical studies.

References

1. Stupphann D, Rauner M, Krenbek D, Patsch J, Pirker T, Muschitz C, Resch H and Pietschmann P: Intracellular and surface RANKL are differentially regulated in patients with ankylosing spondylitis. Rheumatol Int 28: 987-993, 2008.
2. Mattey DL, Packham JC, Nixon NB, Coates L, Creamer P, Hailwood S, Taylor GJ and Bhalla AK: Association of cytokine and matrix metalloproteinase profiles with disease activity and function in ankylosing spondylitis. Arthritis Res Ther 14: R127, 2012.
3. Siebuhr AS, Wang J, Karsdal M, Bay-Jensen AC, Y J and Q Z: Matrix metalloproteinase-dependent turnover of cartilage, synovial membrane, and connective tissue is elevated in rats with collagen induced arthritis. J Transl Med 10: 195, 2012.
4. Hu Z, Lin D, Qi J, Qiu M, Lv Q, Li Q, Lin Z, Liao Z, Pan Y, Jin O, *et al*: Serum from patients with ankylosing spondylitis can increase PPARD, fra-1, MMP7, OPG and RANKL expression in MG63 cells. Clinics (Sao Paulo) 70: 738-742, 2015.
5. Raju R, Balakrishnan L, Nanjappa V, Bhattacharjee M, Getnet D, Muthusamy B, Kurian Thomas J, Sharma J, Rahiman BA, Harsha HC, *et al*: A comprehensive manually curated reaction map of RANKL/RANK-signaling pathway. Database (Oxford) 2011: bar021, 2011.
6. Mou YK, Zhang PP, Li QX, Lin ZM, Liao ZT, Wei QJ and Gu JR: Changes of serum levels of MMP-3, sRANKL, and OPG in juvenile-onset ankylosing spondylitis patients carrying different HLA-B27 subtypes. Clin Rheumatol 34: 1085-1089, 2015.
7. Barrett E, Brothers S, Wahlestedt C and Beurel E: I-BET151 selectively regulates IL-6 production. Biochim Biophys Acta 1842: 1549-1555, 2014.
8. Lamoureux F, Baud'huin M, Rodriguez Calleja L, Jacques C, Berreur M, Rédini F, Lecanda F, Bradner JE, Heymann D and Ory B: Selective inhibition of BET bromodomain epigenetic signalling interferes with the bone-associated tumour vicious cycle. Nat Commun 5: 3511, 2014.
9. Chaidos A, Caputo V, Gouvedenou K, Liu B, Marigo I, Chaudhry MS, Rotolo A, Tough DF, Smithers NN, Bassil AK, *et al*: Potent antimyeloma activity of the novel bromodomain inhibitors I-BET151 and I-BET762. Blood 123: 697-705, 2014.
10. Parkmin KH, Lim E, Lee MJ, Park SH, Giannopoulou E, Yarilina A, van der Meulen M, Zhao B, Smithers N, Witherington J, *et al*: Inhibition of osteoclastogenesis and inflammatory bone resorption by targeting BET proteins and epigenetic regulation. Nat Commun 5: 5418, 2013.
11. Zochling J and Braun J: Assessments in ankylosing spondylitis. Best Pract Res Clin Rheumatol 21: 699-712, 2007.
12. Calin A, Garrett S, Whitelock H, Kennedy LG, O'Hea J, Mallorie P and Jenkinson T: A new approach to defining functional ability in ankylosing spondylitis: The development of the Bath Ankylosing Spondylitis Functional Index. J Rheumatol 21: 2281-2285, 1994.
13. Li X, He L, Hu Y, Duan H, Li X, Tan S, Zou M, Gu C, Zeng X, Yu L, *et al*: Sinomenine suppresses osteoclast formation and Mycobacterium tuberculosis H37Ra-induced bone loss by modulating RANKL signaling pathways. PLoS One 8: e74274, 2013.
14. He L, Duan H, Li X, Wang S, Zhang Y, Lei L, Xu J, Liu S and Li X: Sinomenine down-regulates TLR4/TRAF6 expression and attenuates lipopolysaccharide-induced osteoclastogenesis and osteolysis. Eur J Pharmacol 779: 66-79, 2016.
15. Lin P, Bach M, Asquith M, Lee AY, Akileswaran L, Stauffer P, Davin S, Pan Y, Cambronne ED, Dorris M, *et al*: HLA-B27 and human β2-microglobulin affect the gut microbiota of transgenic rats. PLoS One 9: e105684, 2014.
16. Rysnik O, McHugh K, van Duivenvoorde L, van Tok M, Taurog J, Kollnberger S, Baeten D and Bowness P: Data showing non-conventional HLA-B27 expression in axial joints and gut tissue from B27 transgenic rats, and in frozen and paraffin-fixed synovial SpA tissue. Data Brief 9: 100-111, 2016.
17. Atkinson SM, Bleil J, Maier R, Kühl AA, Thorn M, Serikawa K, Fox B, Kruse K, Haase C, Skov S, *et al*: Anti-RANKL treatment inhibits erosive joint destruction and lowers inflammation but has no effect on bone formation in the delayed-type hypersensitivity arthritis (DTHA) model. Arthritis Res Ther 18: 28, 2016.
18. Reveille JD: Biomarkers for diagnosis, monitoring of progression, and treatment responses in ankylosing spondylitis and axial spondyloarthritis. Clin Rheumatol 34: 1009-1018, 2015.
19. Baud'huin M, Lamoureux F, Jacques C, Rodriguez Calleja L, Quillard T, Charrier C, Amiaud J, Berreur M, Brounais-LeRoyer B, Owen R, *et al*: Inhibition of BET proteins and epigenetic signaling as a potential treatment for osteoporosis. Bone 94: 10-21, 2017.
20. Shaw AT and Gravallese EM: Mediators of inflammation and bone remodeling in rheumatic disease. Semin Cell Dev Biol 49: 2-10, 2015.
21. Im CH, Kang EH, Ki JY, Shin DW, Choi HJ, Chang EJ, Lee EY, Lee YJ, Lee EB, Kim HH and Song YW: Receptor activator of nuclear factor kappa B ligand-mediated osteoclastogenesis is elevated in ankylosing spondylitis. Clin Exp Rheumatol 27: 620-625, 2009.