Bromodomain and Extra-terminal Proteins (BET) Inhibitors Suppress Chondrocyte Differentiation and Restrain Bone Growth

Ningning Niu, Rui Shao, Guang Yan, Weiguo Zou

From the State Key Laboratory of Cell Biology, CAS Center for Excellence in Molecular Cell Sciences, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Running title: BET Inhibitors Induce Cartilage Destruction

To whom correspondence should be addressed: Prof. Weiguo Zou, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, SIBCB Building, Rm 1401, Shanghai, 200031, China, Telephone: 86-21-54921320; Fax: 86-21-54921279; Email: zouwg94@sibcb.ac.cn

Keywords: BET inhibitors, anticancer drug, Col2a1, chondrocyte, cartilage, bone, epigenetics

ABSTRACT

Small-molecule inhibitors for bromodomain and extra-terminal proteins (BET) have recently emerged as potential therapeutic agents in clinical trials for various cancers. However, to date, it is unknown whether these inhibitors have side effects on bone structures. Here, we report that inhibition of BET bromodomain proteins may suppress chondrocyte differentiation and restrain bone growth. We generated a luciferase reporter system using the chondrogenic cell line, ATDC5, in which the luciferase gene was driven by the promoter of Col2a1, an elementary collagen of chondrocyte. The COL2A1-luc ATDC5 system was used for rapidly screening both activators and repressors of human collagen COL2A1 gene expression, and we found that BET bromodomain inhibitors reduce the COL2A1-luc. Consistent with the luciferase assay, BET inhibitors decrease the expression of Col2a1. Furthermore, we constructed a zebrafish line in which the Enhanced Green Fluorescent Protein (EGFP) expression was driven by Col2a1 promoter. The transgenic (Col2a1-EGFP) zebrafish line demonstrated that BET inhibitors I-BET151 and (+)-JQ1 may affect EGFP expression in zebrafish. Furthermore, we found that I-BET151 and (+)-JQ1 may affect chondrocyte differentiation in vitro and inhibit zebrafish growth in vivo. Mechanistic analysis revealed that BET inhibitors influenced the depletion of RNA polymerase II from the Col2a1 promoter. Collectively, these results suggest that BET bromodomain inhibition may have side effects on skeletal bone structures.

Bromodomain and extra-terminal (BET) proteins (containing four mammalian members, BRD2, BRD3, BRD4 and BRDT) function as key epigenetic readers by recognizing histone acetylation through binding to ε-N-lysine acetylation motifs of histone, such as K5, K12 and K16 residues of H4 histone (1, 2), and K27 residues of H3 histone (3). The BET proteins interact with the positive transcription elongation factor (P-TEFβ) and RNA polymerase II (Pol II) to facilitate gene transcription (4-6). A list of drugs, such as I-BET151, I-BET762, PFI-1 and (+)-JQ1 were discovered to target BET proteins and block the interaction between BET proteins and acetyl-lysine of histones (2, 7).

BET proteins and BET inhibitors have been reportedly involved in a variety of biological processes. Pharmacological inhibition of BET proteins lowers the expression of key transcription factors such as oncogene c-MYC (8), clamps the transductions of PI3K Signaling (9), inhibits Gli1 transcription and Hedgehog pathway (10), blocks transcription in neurons (11), represses VEGF-induced angiogenesis and vascular permeability.
(12), reduces cell viability of osteosarcoma cells and inhibits osteoblastic differentiation (13), and restrains osteoclastogenesis (14). Meanwhile, potent BET inhibitors have been identified as showing anti-tumor efficacy in a number of preclinical cancer models in recent years, including leukemia, multiple myeloma, lymphoma, melanoma, and gastric cancer (15,16). This led to clinical studies focusing mostly on the treatment of leukemia and lymphoma. As a result of these studies, the first encouraging signs of efficacy have already been reported (17). Furthermore, previous studies showed that BET inhibitors also control neuronal differentiation and cause an autism-like syndrome (18). However, BET inhibitors were not examined extensively in these studies to determine its side effects on skeletal bone structures.

The long bone is mainly formed through endochondral bone formation, which starts with the formation of a cartilage template from condensed mesenchymal cells. The chondrocytes of the cartilage template proliferate axially, and subsequently undergo hypertrophy and expansion in cellular volume (19). In addition to cartilage development, reports show that the chondrocytes secrete a variety of collagen proteins (20). Type II and X collagens (Col2a1 and Col10a1) contribute to chondrocyte maintenance and proliferation (20,21). Hypertrophic chondrocytes will then switch from the synthesis of the cartilage-characteristic collagens to the synthesis of type I collagen (Col1a1), an osteoblast-characteristic collagen which organizes a mineralizing bone matrix (20,21).

To further examine the underlying mechanisms for chondrocyte proliferation and growth inhibition we established a chondrogenic ATDC5 cell line with the Col2a1 promoter (COL2A1-luc ATDC5 system), and screened a list of epigenetic compounds. Strikingly, six different BET inhibitors were shown to reduce COL2A1-luc, consistent with the decrease of Col2a1 mRNA and protein. Furthermore, we found that BET inhibitors blocked the differentiation of chondrocyte culture. Consistent with this, BET inhibitors I-BET151 and (+)-JQ1 could induce the retardation of the growth of zebrafish. Taken together, these data suggest that the COL2A1-luc ATDC5 system may be used to screen chondrogenic factors. Furthermore, these data suggest that anti-carcinogenic BET inhibitors may potentially injure chondrocytes and the bones of patients.

RESULTS

BET inhibitors suppressed the expression of Col2a1 in chondrogenic ATDC5 cells — Type II collagen is the specific and major matrix protein of cartilage. The Col2a1 promoter has been used to identify the molecules related to the chondrogenesis (22,23). To explore novel activators or repressors of chondrogenic differentiation, we generated an ATDC5 cell line that includes the 3-Kb human Col2a1 promoter ligated to the open reading frame of firefly luciferase (Fig. 1A). We checked the responses of COL2A1-luc to the heterocyclic molecule kartogenin (KGN) and transcriptional factor SOX9, which have been widely recognized to promote the expression of Col2a1 and chondrocyte differentiation (24,25). In agreement with previous reports (24,25), both KGN and SOX9 increased Col2a1 promoter activities in a dose-dependent manner (Fig. 1B and C), suggesting that COL2A1-luc ATDC5 system could be used to screen chondrogenic activators or repressors.

Epigenetic factors (i.e., chromatin modifier) regulate normal and disease processes, and is mediated in part by the methylation status of DNA as well as by chemical modifications of histones, including acetylation, methylation, phosphorylation, and ubiquitination (26). Chondrogenic processes are also influenced by epigenetic regulation, such as methylation of K27 residues on histone H3 (27). In order to excavate more epigenetic regulators, we screened a collection of 38 chemicals involved in epigenetic regulations using the COL2A1-luc ATDC5 system (Table 1). After 48 h treatment in 10 μM final concentration of BET inhibitors, we found that a battery of epigenetic inhibitors decreased luciferase activities of Col2a1 promoter (Fig. 2A). Among them, all BET inhibitors, including Bromosporine, I-BET-762, RVX208, I-BET151, PFI-1 and (+)-JQ1, could significantly suppress the luciferase activity of the Col2a1 promoter (Fig. 2A), compared with the non-response of the...
BET Inhibitors Induce Cartilage Destruction

promoter of a soybean translation elongation factor EF1 alpha (EF1α) (Fig. 2B).

Consistent with the luciferase activity result, the RNA levels of Col2a1 were also significantly reduced in cells treated with BET inhibitors compared with vehicle alone (DMSO) (Fig. 2C). Consistently, we discovered that BET inhibitors decreased Col2a1 expression in mesenchymal progenitor cell line, C3H/10T1/2 (C3H10) (Fig. 2D–F) and primary chondrocytes (Fig. 2G–I).

The half maximal inhibitory concentration and cytotoxicity concentration were unequal for different BET inhibitors. To lower the impact of cell cytotoxicity of the various BET inhibitors, we further examined the effects of five BET inhibitors (I-BET-762, RVX208, I-BET151, PFI-1 and (+)-JQ1) on Col2a1 expression at various doses. We performed the treatment of five BET inhibitors at gradient concentrations of 0.1, 1 and 5 μM. As shown in Figure 3A to E (ATDC5) and Figure 3F to J (C3H10), all of the BET inhibitors were capable of dose-dependently decreasing the luciferase activity of the Col2a1 promoter. Consistent with this, the expression level of Col2a1, as determined by quantitative RT-PCR, were dose-dependently reduced by various BET inhibitors (Fig. 3K–O in ATDC5, and Fig. 3P–T in primary chondrocytes).

BET inhibitors does-dependently suppressed the expression of chondrogenic marker genes in primary chondrocytes — BET bromodomain inhibition results in the suppression of Col2a1, which is one of the most essential collagens expressed during cartilage maintenance and differentiation. We hypothesized that BET inhibitors may also play fundamental roles in chondrocyte proliferation and development. To test our hypothesis, we isolated chondrocytes from the articular cartilage of 3-day-old wild-type mice, and examined the effects of BET bromodomain inhibition on the expression of chondrocyte marker genes. Total RNA and protein were collected from the cultured chondrocytes that were treated with I-BET151 and (+)-JQ1 at a final concentration of 0.2, 1 and 5 μM (I-BET151) and 0.2, 0.5 and 1 μM (+)-JQ1) for 72 h. Using quantitative RT-PCR, we observed a progressively transcription suppression of Col2a1, a disintegrin and metallopeptidase with thrombospondin motifs 5 (Adamts5) and matrix metalloproteinase-3 (Mmp-3) (Fig. 4A–F). However, the key transcriptional factor of chondrogenesis, Sox9, showed no obvious response to the BET bromodomain inhibition (data not shown). We also examined the protein level of different chondrocyte marker genes after I-BET151 and (+)-JQ1 treatment. As shown in Figure 4G and H, immunoblot analysis demonstrated both BET151 and (+)-JQ1 may lead to decreased protein levels of COL2A1, ADAMTS5 and MMP3, correlating with RNA level inhibition. These data indicated that both I-BET151 and (+)-JQ1 treatment may have suppressive effects on chondrogenesis.

BET inhibition with I-BET151 and (+)-JQ1 arrests chondrogenesis and differentiation — To further characterize the efficacy of inhibiting BET proteins in chondrogenic differentiation, we examined the effect of I-BET151 and (+)-JQ1 by using the chondrocyte micromass culture system. Inhibition of BET bromodomain proteins induced a remarkable decrease in matrix accumulation, which was monitored by alcian blue staining after 7 days in micromass culture (Fig. 5A). Accordingly, both I-BET151 and (+)-JQ1 strongly suppressed the expression of chondrogenesis-related genes, such as Col2a1, Adamts5 and Mmp3 (Fig. 5B–D). Consistently, the chondrogenic differentiation of C3H10 and ATDC5 cells were inhibited by I-BET151 and (+)-JQ1 as well (Fig. 5E and F).

Next, we examined the effects of BET inhibitors on ex vivo mice articular cartilage cultures. Articular cartilage of distal femurs was isolated from newborn mice and cultured in chondrogenic differential medium. I-BET151 at 5 μM or (+)-JQ1 at 0.5 μM was added to the culture medium with equal amount of DMSO as the control. After 1 week of culture, we observed that both I-BET151 and JQ1 could suppress the growth of cartilages, as the size of the I-BET151 and JQ1 treatment group is significantly smaller than the DMSO control group (Fig. 5G–J). We examined the cartilage by positive safranin O staining and found that both I-BET151 and (+)-JQ1 can inhibit the hypertrophy of chondrocytes as determined by the size of the chondrocytes following treatment (Fig. 5G and H).
I-BET151 and (+)-JQ1 modulated the accumulation of Pol II on Col2a1 promoter — BET inhibitors are capable of blocking the interaction between acetylated lysine with BET proteins, which could regulate gene transcription driven by Pol II (28). Firstly, we detected that the protein level of Pol II was not impacted by BET inhibitors’ treatment (Fig. 6A). We next examined whether BET inhibitors could affect the recruitment of Pol II to the Col2a1 promoter. For the 2.5 kb promoter upstream of the transcription start site of mouse Col2a1, we analyzed four loci (Fig. 6B, primers 1 – 4). DNA was purified after chromatin immunoprecipitation with Pol II antibody in primary chondrocytes treated with DMSO, I-BET151 or (+)-JQ1 for 72 h. The relative amount of promoter-associated Pol II was evaluated by quantitative RT-PCR followed by normalization with the IgG sample. We observed that Pol II associates with the Col2a1 promoter, especially DNA fragments close to the transcription start site (TSS) (Fig. 6C). In addition, the association between Pol II and the Col2a1 promoter was arrested by BET bromodomain inhibition by I-BET151 and (+)-JQ1 (Fig. 6C). These data indicate that the recruitment of Pol II to the Col2a1 promoter was interrupted by BET inhibitors.

I-BET151 and (+)-JQ1 retarded the growth of zebrafish — Zebrafish is a wildly used good model in developmental and functional studies of bone and cartilage (29). To determine whether BET inhibitors could influence Col2a1 as well as animal growth in vivo, we next examined the effects of BET inhibitors on zebrafish. We generated a transgenic zebrafish line harboring a Col2a1 promoter-EGFP transgene (Fig. 7A). As shown in Figure 7B, the EGFP fluorescence could be detected at 1 dpf (days post-fertilization), and was further enhanced at 2 dpf. The EGFP fluorescence localized at the vertebrate, which contains Collagen 2 positive chondrocytes. We incubated 12 hr post-fertilization embryos with different doses of I-BET151 (1, 5 and 10 μM) and (+)-JQ1 (0.1, 0.5 and 1 μM) for 36 hours. As shown in Figure 7C and D, 10 μM I-BET151 and 1 μM (+)-JQ1 induced serious malformations in the zebrafish. In addition, both I-BET151 and (+)-JQ1 led to decreased EGFP fluorescence compared to the vehicle alone (DMSO), indicating that BET inhibitors could decrease Col2a1 expression in vivo (Fig. 7C and D).

Prior research studies demonstrate that long bones are formed through endochondral ossification of chondrocytes in which Col2a1 is one of the most requisite matrix collagens (30). Thus, we hypothesized that the inhibition of BET bromodomain proteins may also affect the growth of the organism. To test this hypothesis, we incubated 2 dpf embryos with I-BET151 (1 and 5 μM) and (+)-JQ1 (0.1 and 0.5 μM) for two weeks and measured the body length of the zebrafish following two weeks of cultivation. As shown in Figure 7E and F, zebrafish treated with I-BET151 at both 1 and 5 μM developed a specified amount of atrophy compared with vehicle alone (DMSO). However, I-BET151 at high concentrations (10 μM) impacted zebrafish viability (Fig. 7G). Consistently, we also observed that 0.5 μM (+)-JQ1 suppressed the length extension of the zebrafish, while 0.1 μM (+)-JQ1 showed no obvious functions (Fig. 7H–J). Next, we characterized the whole mount skeleton of zebrafish by alizarin red and alcian blue staining at 3-wk-old, and observed similar shortened body length (Fig. 7K and L). In order to analyze the bone growth restriction, we measured the length of centra 15 (c15) of caudal vertebrae representative, and observed the shorter vertebrae bodies in I-BET151 (both 1 and 5 μM) and (+)-JQ1 (0.5 μM) treated zebrafish, while 0.1 μM (+)-JQ1 showed no obvious functions (Fig. 7M–P).

To demonstrate that BET inhibitors also played fundamental roles in zebrafish chondrogenesis, we isolated total RNAs of tail fins from 3-wk-old zebrafish under different treatments. Using quantitative RT-PCR, we observed a progressively transcription suppression of cartilage and bone marker genes, such as Col2a1, Adamts5, Col11a1 (Type I Collagen), Runx2a and Runx2b (runt-related transcription factor 2a or b) (Fig. 8A–J). Consistent with this, the protein level of COL2A1 was reduced in paraffin sections of 3-wk-old zebrafish treated with both I-BET151 and (+)-JQ1 (Fig. 8K). Taken together, our data suggest that BET inhibitors restrained chondrogenesis and differentiation both in vitro and in vivo.
DISCUSSION

In the present study, we generated a Col2-luc ATDC5 system to identify activators or repressors of chondrogenic differentiation. We found that six BET inhibitors restrain the expression of Col2α1 and impede chondrogenesis as determined by micromass culture and ex vivo cartilage culture. We also established a Col2-eGFP transgenic zebrafish line and demonstrated that BET inhibitors can decrease Col2-eGFP expression. Consistently, BET inhibitors retard the growth of zebrafish in vivo. These data demonstrate that BET inhibitors could inhibit chondrogenic differentiation and retain bone growth in vitro and in vivo.

The BET subfamily of bromodomain-containing proteins are involved in a number of hematological and solid tumors (15,17). Currently, several active clinical trials aiming to treat malignancies have been proceeded using BET inhibitors, such as FT-1101, ZEN003694, BMS-986158, INCB054329, RVX-208, I-BET 762, OTX 015, CPI-0610 and TEN-010 (https://www.clinicaltrials.gov). In addition to this, multiple other BET inhibitors have been well studied and have revealed great potential for clinical application. For instance, (+)-JQ1 suppresses breast cancer cells (31), gastric cancer cells (32), acute myeloid leukemia cells (33) and osteosarcoma cells (13). Furthermore, I-BET151 has been shown to induce cell apoptosis in myeloma and leukemia (16,34). However, because BET bromodomain proteins function as epigenetic regulators and play a key role in transcriptional regulation of essential genes in cell cycle and apoptosis, such as c-Myc (8), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) (35) and B-cell lymphoma 2 (BCL2) (8,36), the inhibitory factors may affect other important biological processes.

Our current study demonstrated that BET inhibitors could interfere with the chondrogenic differentiation and bone growth, highlighting the necessity of studying the role of bromodomain-containing proteins in chondrogenesis to monitor and avoid possible side effects of BET inhibitors on cartilage and bone. Besides bone formation, bone homeostasis is maintained by the balance between osteoblastic bone formation and osteoclastic bone resorption, while the growth of long bones is from endochondral ossification of chondrocytes derived from cartilage (15). Previous studies have shown that BET inhibitors may affect osteoclastogenesis (14) and bone-associated tumor vicious cycle (13). In this study, we demonstrated BET inhibitors as repressors of chondrogenesis. Taken together, previous studies in addition to our studies demonstrate that preclinical pharmacological studies of BET inhibitors on human bone warrant further investigation.

EXPERIMENTAL PROCEDURES

Cell culture — Mouse embryonic cell line ATDC5 was maintained in DMEM (Corning) that contains the following Ham’s F12 (1:1) (Corning), 2 mM glutamine and 5% fetal bovine serum (FBS). Mouse mesenchymal stem cell line (C3H/10T1/2) were obtained from ATCC and maintained in low glucose alpha MEM containing 10% FBS. The cells were plated at 1.5 x 10^6 per 10-cm-dish. To acquire primary chondrocytes, articular cartilage from 3-day-old wild-type mice were isolated and digested for 1 h in enzymatic hydrolysate, which contains the following: 50 ml low glucose alpha MEM (Corning), 100 ng Dispase II (D4693, Sigma–Aldrich, St. Louis, Missouri, United States of America) and 50 mg collagenase (C0130, Sigma). For chondrogenesis cultures and alcian blue staining, chondrocytes were centrifuged at 300 xg for 5 min, and cultured in low glucose alpha MEM containing 10% FBS.

Culture and differentiation of chondrocyte cells — Two-dimensional-micromass culture was initiated by spotting 2.5x10^5 chondrocytes in 15 μl to a well of 12-well-plate and maintained in the chondrogenic differentiation medium: DMEM supplemented with 1% ITS, 500 nM/ml TGFβ3 (R&D Systems, 243-B3-200), 10 nM dexamethasone, 10 mM β-glycerophosphate and 0.1 mM L-ascorbic-acid-2-phosphate. After a 7-day differentiation, the micromass culture was stained with 1% alcian blue. For cartilage-like particle differentiation, articular cartilages were isolated from distal femurs of newborn wild-type mice and cultured in chondrogenic differentiation medium and growth medium (low glucose alpha MEM containing 10% FBS) for 7 days.
Phenotype analyses — For histology analyses, cartilage-like particles were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated using graded ethanol overnight, and embedded in paraffin. The paraffin-embedded tissue samples were sectioned at 5 μm. For safranin O staining, sections were firstly dewaxed, immersed in 1% acetic acid, and stained with 0.05% fast green and 0.5% safranine 1%. Immunohistochemistry staining was performed as previously described (37). Images were captured using a microscope (Olympus BX51). Alizarin red and alcian blue staining was performed as previously described (38).

Zebrafish were maintained as described (39). For compound treatment, DMSO and BET inhibitors were added as indicated at 1 day(s) post-fertilization (dpf). Images were captured using Olympus BZX16 stereomicroscope.

Luciferase reporter assays — ATDC5 cells grown in 10-cm-dishes were transiently transfected using polyethylenimine (Sigma–Aldrich, 765090) with the luciferase reporter constructs. The pRL-TK (Promega) was co-transfected provided a normalization control for transfection efficiency. Eight hours after transfection, ATDC5 cells with luciferase and control Renilla were digested and plated at 1.5 x 10^5 per well in 12-well-plate. Four hours later, cells were treated with different compounds. I-BET151 and (+)-JQ1 was firstly dissolved to DMSO at 5 mM, and then diluted to final concentration as described. The vehicle, DMSO was added as blank control. After 48-h cultivation, cells were lysed and luciferase activity was measured using the Dual Luciferase Assay Kit (Promega, Madison, Wisconsin, United States of America). Cells were lysed and luciferase activity was measured using the Dual Luciferase Assay Kit (Promega).

Reverse transcription and real-time PCR — Total RNA was prepared from cells or tissues using TRIzol (T9424, Sigma), and reverse transcribed into cDNA using PrimeScript™ RT Reagent Kit (PR037A, Takara Bio Inc., Kusatsu, Siga, Japan). The cDNA quantity was analyzed by the BioRad CFX96 system (BioRad, Hercules, California, United States of America). The primer sets used were as follows: for mouse genes, β-ACTin sense 5’-AGATGTGGATCAGCAAGCAG-3’ and antisense 5’-GCAGAAGTCTTTGTTTGGTC-3’; Col2a1 sense 5’-CGGTTCCTACGGTGTCAGG-3’ and antisense 5’-GCAGAGGACATTTCCAGGTG-3’; Adamts5 sense 5’-GACACAGTGCTGGAGGTAGCA-3’ and antisense 5’-CGTCATGAGAAAGGCCCAGTAG-3’; Mmp3 sense 5’-GACGATGATGAACTGAGTGGACAG-3’ and antisense 5’-TTGAGAGAGATGGAACCGGC-3’; for zebrafish genes, Actin β1 sense 5’-TCTTGTTGATGGAATCTCGG-3’ and antisense 5’-ACGGTGCTCAATGCAAGTTGA-3’; Col2a1 sense 5’-CCCTGAATGGAAGAGCGGTG-3’ and antisense 5’-TGCTTTGTGTCCTCACCAGTTC-3’; Col1a1 sense 5’-AACTCCCTGAGCCAGCAGAT-3’ and antisense 5’-TGACGCAATCTCGGAGCAG-3’; Acan sense 5’-TCTGAGAGGATGCGCTTGTGA-3’ and antisense 5’-TGGCCTTCTCCACTGTCCGTCT-3’; Adamt35 sense 5’-CGCTCAACCGCTCTGTCCTCAAC-3’ and antisense 5’-TCCTGCCTTGGGAGTGGTA-3’; Mmp13a sense 5’-CGACGCAGTCTCCTCTACAAGGAAG-3’ and antisense 5’-ATACTGGAAGGCTGCGTGAC-3’; Mmp13b sense 5’-GACGCTGCCCTATGAGAACCCA-3’ and antisense 5’-GATCGTGGATCAGCAAGCAG-3’; Ocn sense 5’-TCTTGTTGATGGAATCTCGG-3’ and antisense 5’-TGCTTTGTGTCCTCACCAGTTC-3’; Ocn sense 5’-GAGATAGACACATCGAGGCAAAT-3’ and antisense 5’-TCCTCAGACTCCACTTTCTTCC-3’; Bsp sense 5’-AGGCCTATTCCAGCACCA-3’ and antisense 5’-AGGGATGATGATTCCACAGAC-3’; Runx2a sense 5’-CCAGCAACATTCACCTACCAACC-3’ and antisense 5’-TATGGTGCTGCTGGTTTGGAA-3’; Runx2b sense 5’-CGCCAGTTCCCTGCGCTT3-3’ and antisense 5’-CGAGGAAGCACCCTAATAGAG-3’;

Western blotting — Cell extracts were prepared with EBC buffer (50 mM Tris–HCl [pH
7.5], 120 mM NaCl, 0.5% NP40, protease inhibitor cocktail (Sigma–Aldrich, S8820)). Protein lysates (20 μg) were separated on 10% SDS–polyacrylamide gels, transferred onto PVDF membranes, blocked with 5% blotting grade milk in Tris-buffered saline with tween (20 mM Tris–HCl [pH 7.6], 137 mM NaCl, 0.5% Tween 20), probed with the indicated primary antibodies, reacted with corresponding secondary antibodies, and detected using a chemiluminescence assay (EMD Millipore, Billerica). Membranes were exposed to X-ray film (Amersham Pharmacia Biotech, Piscataway, New Jersey, United States of America) for visualization.

ChIP assay — About 10^7 chondrocytes were cultured with 1 μM I-BET151, 0.1 μM (+)-JQ1 or DMSO added. After 72-h treatment, cells were resuspended in IP buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 1mM PMSF) and subjected to gentle sonication and centrifuged (12000 xg, 10 min) to obtain cell extracts. ChIP was performed as described (40). IgG (Sigma–Aldrich, 15006) and RNA polymerase II antibody (EMD Millipore, 05-623) were used at 1 μg per 10^6 cells.

Plasmids and PCR primers — A 3-Kb Col2a1 region was obtained from total mouse genome using primers: Col2-F: 5'-TTAATTCTAGTTAGTGACTTCTATCCATGTTGTTTGGCTATATTGCTATAATGGTTTACAAATAAA GCAATAGCATCACAATTTCACAAATAAAGTGTATTTCCTACTGATTTAGTTGTTGTTGTTGTTGTTGTTGTTG TTGTCCAAACTCATCAATGTATCTTAAAGGCC TAAATTGTAGACGTT. To generate the HA-SOX9 construct, SOX9 was amplified from the total cDNA library of 10.5-day-old embryonic mice (E10.5) using primers SOX9-F: 5'-GGCGCCGATGAATCTCTGGGCCCTTCA TGA-3' and SOX9-R: 5'- GGATCTCTAGGTCGATGCTGTGGTGGTGTA G-3', and cloned into the modified pHAGE vector containing HA using restriction enzymes Not I and BamH I. To generate Col2a1-eGFP construct, 1.9-Kb Col2a1 promoter region was obtained from total mouse genome using primers: Col2a1-5-xho1: 5'-AATTAACCTGAGGCGCCTTCTGACACTGTA TGCCAATT-3' and Col2a1-3-sma1: 5'-AATTAACCGGGGTCTAAAGATTAGACAT GCAGGT-3', and inserted into plasmid pEsecl.

Antibodies — Anti-HA antibody (SC-7392) was from Santa Cruz Biotech; anti-GFP antibody (66002-1) was from Proteintech; anti-Col2a1 antibody (ab34712), anti-Mmp3 antibody (ab52915), monoclonal anti-Histone H3 antibody (ab1791) and anti-Tubulin antibody (SC-23948) were from Santa Cruz Biotechnology; anti-Adams5 antibody (PA5-14350) was from Thermo Scientific.

Statistical analysis — For statistical analysis, in most graphs mean values with standard deviation (SD) are shown. Data were generated from several independently obtained data sets. P values were obtained from two-tailed student t-tests. A P value less than 0.05 was considered significant difference, and less than 0.01 was considered extremely significant difference.

Acknowledgments: We thank the members of the Zou lab for valuable discussions.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: W. Z. and N. N. conceived and supervised the project. As shown in Figure 1, R. S. and N. N. generated the COL2A1-luc ATDC5 system and performed the screening of the epigenetic compounds. G. Y. generated transgenic zebrafish line Tg (Col2-EGFP) and contributed to the preparation
of Figure 7. N. N. performed other experiments and data analysis. N. N. and W. Z prepared the manuscript.

REFERENCES

1. Leroy, G., Rickards, B., and Flint, S. J. (2008) The double bromodomain proteins Brd2 and Brd3 couple histone acetylation to transcription. *Molecular cell* 30, 51-60
2. Filippakopoulos, P., and Knapp, S. (2014) Targeting bromodomains: epigenetic readers of lysine acetylation. *Nature reviews. Drug discovery* 13, 337-356
3. Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K., and Zhou, M. M. (1999) Structure and ligand of a histone acetyltransferase bromodomain. *Nature* 399, 491-496
4. Whyte, W. A., Orlando, D. A., Hnisz, D., Abraham, B. J., Lin, C. Y., Kagey, M. H., Rahl, P. B., Lee, T. I., and Young, R. A. (2013) Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* 153, 307-319
5. Yang, Z., Yik, J. H., Chen, R., He, N., Jang, M. K., Ozato, K., and Zhou, Q. (2005) Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Molecular cell* 19, 535-545
6. Jang, M. K., Mochizuki, K., Zhou, M., Jeong, H. S., Brady, J. N., and Ozato, K. (2005) The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. *Molecular cell* 19, 523-534
7. Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W. B., Fedorov, O., Morse, E. M., Keates, T., Hickman, T. T., Felletar, I., Philpott, M., Munro, S., McKeown, M. R., Wang, Y., Christie, A. L., West, N., Cameron, M. J., Schwartz, B., Heightman, T. D., La Thangue, N., French, C. A., Wiest, O., Kung, A. L., Knapp, S., and Bradner, J. E. (2010) Selective inhibition of BET bromodomains. *Nature* 468, 1067-1073
8. Delmore, J. E., Issa, G. C., Lemieux, M. E., Rahl, P. B., Shi, J., Jacobs, H. M., Kastritis, E., Gilpatrick, T., Paranal, R. M., Qi, J., Chesi, M., Schinzel, A. C., McKeown, M. R., Hefferman, T. P., vakoc, C. R., Bergsagel, P. L., Ghobrial, I. M., Richardson, P. G., Young, R. A., Hahn, W. C., Anderson, K. C., Kung, A. L., Bradner, J. E., and Mitsiades, C. S. (2011) BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 146, 904-917
9. Stratikopoulos, E. E., Dendy, M., Szabolcs, M., Khaykin, A. J., Lefebvre, C., Zhou, M. M., and Parsons, R. (2015) Kinase and BET Inhibitors Together Clamp Inhibition of PI3K Signaling and Overcome Resistance to Therapy. *Cancer cell* 27, 837-851
10. Tang, Y., Gholamin, S., Schubert, S., Willardson, M. I., Lee, A., Bandopadhayay, P., Berghold, G., Masoud, S., Nguyen, B., Yue, N., Balansay, B., Yu, F., Oh, S., Woo, P., Chen, S., Ponnuswami, A., Monje, M., Atwood, S. X., Whitson, R. J., Mitra, S., Cheshier, S. H., Qi, J., Beroukhim, R., Tang, J. Y., Wechsler-Reya, R., Oro, A. E., Link, B. A., Bradner, J. E., and Cho, Y. J. (2014) Epigenetic targeting of Hedgehog pathway transcriptional output through BET bromodomain inhibition. *Nature medicine* 20, 732-740
11. Korb, E., Herre, M., Zucker-Scharff, I., Darnell, R. B., and Allis, C. D. (2015) BET protein Brd4 activates transcription in neurons and BET inhibitor Jq1 blocks memory in mice. *Nature neuroscience* 18, 1464-1473
12. Huang, M., Qiu, Q., Xiao, Y., Zeng, S., Zhan, M., Shi, M., Zhou, Y., Ye, Y., Liang, L., Yang, X., and Xu, H. (2016) BET Bromodomain Suppression Inhibits VEGF-induced Angiogenesis and Vascular Permeability by Blocking VEGFR2-mediated Activation of PAK1 and eNOS. *Scientific reports* 6, 23770
13. Lamoureux, F., Baud'huin, M., Rodriguez Calleja, L., Jacques, C., Berreur, M., Redini, F., Lecanda, F., Bradner, J. E., Heymann, D., and Ory, B. (2014) Selective inhibition of BET bromodomain epigenetic signalling interefers with the bone-associated tumour vicious cycle. *Nature communications* 5, 3511
14. Park-Min, K. H., Lim, E., Lee, M. J., Park, S. H., Giannopoulou, E., Yarilina, A., van der Meulen, M., Zhao, B., Smithers, N., Witherington, J., Lee, K., Tak, P. P., Prinjha, R. K., and Ivashikv, L. B. (2014) Inhibition of osteoclastogenesis and inflammatory bone resorption by targeting BET proteins and epigenetic regulation. *Nature communications* **5**, 5418

15. Fu, L. L., Tian, M., Li, X., Li, J. J., Huang, J., Ouyang, L., Zhang, Y. H., and Liu, B. (2015) Inhibition of BET bromodomains as a therapeutic strategy for cancer drug discovery. *Oncotarget* **6**, 5501-5516

16. Fong, C. Y., Gilan, O., Lam, E. Y. N., Rubin, A. F., FOUNI, S., Tyler, D., Stanley, K., Sinha, D., Yeh, P., Morison, J., Giotopoulos, G., Lugo, D., Jeffrey, P., Lee, S. C. W., Carpenter, C., Gregory, R., Ramsay, R. G., Lane, S. W., Abdel-Wahab, O., Kouzarides, T., Johnstone, R. W., Dawson, S. J., Huntly, B. J. P., Prinjha, R. K., Papenfuss, A. T., and Dawson, M. A. (2015) BET inhibitor resistance emerges from leukaemia stem cells. *Nature* **525**, 538-542

17. Jung, M., Gelato, K. A., Fernandez-Montalvan, A., Siegel, S., and Haendler, B. (2015) Targeting BET bromodomains for cancer treatment. *Epigenomics-Uk* **7**, 487-501

18. Sullivan, J. M., Badimon, A., Schaefer, U., Ayata, P., Gray, J., Chung, C. W., von Schimmelmann, M., Zhang, F., Garton, N., Smithers, N., Lewis, H., Tarakhovsky, A., Prinjha, R. K., and Schaefer, A. (2015) Autism-like syndrome is induced by pharmacological suppression of BET proteins in young mice. *Journal of Experimental Medicine* **212**, 1771-1781

19. Mackie, E. J., Tatarczuch, L., and Mirams, M. (2011) The skeleton: a multi-functional complex organ. The growth plate chondrocyte and endochondral ossification. *J Endocrinol* **211**, 109-121

20. Seghatoleslami, M. R., Lichtler, A. C., Upholt, W. B., Kosher, R. A., Clark, S. H., Mack, K., and Rowe, D. W. (1995) Differential regulation of COL2A1 expression in developing and mature chondrocytes. *Matrix Biol* **14**, 753-764

21. Bianco, P., Cancetta, F. D., Riminucci, M., and Cancetta, R. (1998) Bone formation via cartilage models: The “borderline” chondrocyte. *Matrix Biol* **17**, 185-192

22. Zhou, G., Lefebvre, V., Zhang, Z. P., Eberspaecher, H., and de Crombrugghe, B. (1998) Three high mobility group-like sequences within a 48-base pair enhancer of the Col2a1 gene are required for cartilage-specific expression in vivo. *J Biol Chem* **273**, 14989-14997

23. Kan, A., Ikeda, T., Saito, T., Yano, F., Fukai, A., Hojo, H., Ogasawara, T., Ogata, N., Nakamura, K., Chung, U., and Kawaguchi, H. (2009) Screening of Chondrogenic Factors With a Real-Time Fluorescence-Monitoring Cell Line ATDC5-C2ER Identification of Sorting Nexin 19 as a Novel Factor. *Arthritis Rheum-Uk* **60**, 3314-3323

24. Johnson, K. A. (2013) A Stem Cell-Based Approach to Cartilage Repair. *Osteoarthritis Cartilage* **21**, S4-S4

25. Bell, D. M., Leung, K. K. H., Wheatley, S. C., Ng, L. J., Zhou, S., Ling, K. W., Sham, M. H., Koopman, P., Tam, P. P. L., and Cheah, K. S. E. (1997) SOX9 directly regulates the type-II collagen gene. *Nat Genet* **16**, 174-178

26. Kouzarides, T. (2007) Chromatin modifications and their function. *Cell* **128**, 693-705

27. Yapp, C., Carr, A. J., Price, A. J., Oppermann, U., and Snelling, S. J. (2016) H3k27me3 Demethylases Regulate in Vitro Chondrogenesis and Chondrocyte Activity in Osteoarthritis. *Osteoarthritis Cartilage* **24**, S181-S181

28. Zhang, W. S., Prakash, C., Sum, C., Gong, Y., Li, Y. H., Kwok, J. J. T., Thiessen, N., Pettersson, S., Jones, S. J. M., Knapp, S., Yang, H., and Chin, K. C. (2012) Bromodomain-containing Protein 4 (BRD4) Regulates RNA Polymerase II Serine 2 Phosphorylation in Human CD4+T Cells. *J Biol Chem* **287**, 43137-43155

29. Fisher, S., Jagadeeswaran, P., and Halpern, M. E. (2003) Radiographic analysis of zebrafish skeletal defects. *Dev Biol* **264**, 64-76

30. Wei, X., Hu, M., Mishina, Y., and Liu, F. (2016) Developmental Regulation of the Growth Plate and Cranial Synchondrosis. *Journal of dental research*

31. Feng, Q., Zhang, Z., Shea, M. J., Creighton, C. J., Coarfa, C., Hilsenbeck, S. G., Lanz, R., He, B., Wang, L., Fu, X. Y., Nardone, A., Song, Y. C., Bradner, J., Mitsiades, N., Mitsiades, C. S.,
Osborne, C. K., Schiff, R., and O’Malley, B. W. (2014) An epigenomic approach to therapy for tamoxifen-resistant breast cancer. *Cell Res* **24**, 809-819

32. Montenegro, R. C., Clark, P. G., Howarth, A., Wan, X., Ceroni, A., Siejka, P., Nunez-Alonso, G. A., Monteiro, O., Rogers, C., Gamble, V., Burbano, R., Brennan, P. E., Tallant, C., Ebner, D., Fedorov, O., O’Neill, E., Knapp, S., Dixon, D., and Muller, S. (2016) BET inhibition as a new strategy for the treatment of gastric cancer. *Oncotarget*

33. Fiskus, W., Sharma, S., Qi, J., Shah, B., Devaraj, S. G. T., Leveque, C., Portier, B. P., Iyer, S., Bradner, J. E., and Bhatla, K. N. (2014) BET Protein Antagonist JQ1 Is Synergistically Lethal with FLT3 Tyrosine Kinase Inhibitor (TKI) and Overcomes Resistance to FLT3-TKI in AML Cells Expressing FLT-ITD. *Mol Cancer Ther* **13**, 2315-2327

34. Chaidos, A., Caputo, V., Gouvedenou, K., Liu, B. B., Marigo, I., Chaudhry, M. S., Rotolo, A., Tough, D. F., Smithers, N. N., Bassil, A. K., Chapman, T. D., Harker, N. R., Barbash, O., Tummino, P., Al-Mahdi, N., Haynes, A. C., Cutler, L., Le, B. C., Rahemtulla, A., Roberts, I., Kleijnen, M., Witherington, J. J., Parr, N. J., Prinjha, R. K., and Karadimitris, A. (2014) Potent antmyeloma activity of the novel bromodomain inhibitors I-BET151 and I-BET762. *Blood* **123**, 697-705

35. Ceribelli, M., Kelly, P. N., Shaffer, A. L., Wright, G. W., Xiao, W., Yang, Y., Mathews Griner, L. A., Guha, R., Shinn, P., Keller, J. M., Liu, D., Patel, P. R., Ferrer, M., Joshi, S., Nerle, S., Sandy, P., Normant, E., Thomas, C. J., and Staudt, L. M. (2014) Blockade of oncogenic IkappaB kinase activity in diffuse large B-cell lymphoma by bromodomain and extraterminal domain protein inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 11365-11370

36. Asangani, I. A., Dommeti, V. L., Wang, X., Malik, R., Cieslik, M., Yang, R., Escara-Wilke, J., Wilder-Romans, K., Dhanireddy, S., Engelke, C., Iyer, M. K., Jing, X., Wu, Y. M., Cao, X., Qin, Z. S., Wang, S., Feng, F. Y., and Chinnaiyan, A. M. (2014) Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. *Nature* **510**, 278-282

37. Wein, M. N., Jones, D. C., Shim, J. H., Aliprantis, A. O., Sulyanto, R., Lazarevic, V., Poliachik, S. L., Gross, T. S., and Glimcher, L. H. (2012) Control of bone resorption in mice by Schnurri-3. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 8173-8178

38. Liu, Z., Yao, X., Yan, G., Xu, Y. C., Yan, J., Zou, W. G., and Wang, G. (2016) Mediator MED23 cooperates with RUNX2 to drive osteoblast differentiation and bone development. *Nature communications* **7**

39. Fenaroli, F., Westmoreland, D., Benjaminsen, J., Kolstad, T., Skjeldait, F. M., Meijer, A. H., van der Vaart, M., Ulanova, L., Roos, N., Nystrom, B., Hildahl, J., and Griffiths, G. (2014) Nanoparticles as Drug Delivery System against Tuberculosis in Zebrafish Embryos: Direct Visualization and Treatment. *Acs Nano* **8**, 7014-7026

40. Adamo, A., Sese, B., Boue, S., Castano, J., Paramonov, I., Barrero, M. J., and Belmonte, J. C. I. (2015) LSD1 regulates the balance between self-renewal and differentiation in human embryonic stem cells. *Nat Cell Biol* **13**, 652-U265

**FOOTNOTES**

This work was supported in part by grants from the 973 Program from the Chinese Ministry of Science and Technology (MOST) (grant numbers 2015CB64503 and 2014CB964704), and the National Natural Science Foundation of China (NSFC) (grant numbers 31371463 and 31501170). W. Z. is a scholar of the National 1000 Young Talents Program of China and the National Science Fund for Excellent Young Scholars (grant number 81322027).

The abbreviations used are: BET, Bromodomain and extra-terminal; KGN, kartogenin; SD, standard deviation; dpf, days post-fertilization; hpf, hr post-fertilization; TSS, transcription start site; ChiP, Chromatin Immunoprecipitation.
FIGURE LEGENDS

FIGURE 1. Col2a1 promoter luciferase reporter. A, schematic diagram of the Col2a1 promoter luciferase reporter construct. B, relative luciferase activity in cells treated with gradient kartogenin (KGN) in µM. The KGN values 0.1, 1 and 10 are reported as 0.1, 1 and 10 µM, respectively. C, top, relative luciferase activity in cells transfected with gradient Sox9. Bottom, western blot analysis. For B and C, values represent mean ± SD (n = 3). P values were obtained from t tests with paired or unpaired samples, *P < 0.05, **P < 0.01.

FIGURE 2. Screen of epigenetic compounds that influence Col2a1 transcription by luciferase reporter assay. A, the effect of epigenetic compounds was assessed by luciferase activity analysis. All of the epigenetic compounds were added to achieve a final concentration of 10 µM. B, E and H, luciferase activity analysis of EF1α promoter in ATDC5 (B), C3H10 (E) and primary chondrocytes (H). All of epigenetic compounds were added to 10 µM final concentration. C, F and I, analysis of Col2a1 transcriptional levels by quantitative RT-PCR using RNA isolated from ATDC cells (C), C3H10 cells (F) and primary chondrocytes (I) treated with 1 µM BET inhibitors respectively. D and G, luciferase activity analysis of COL2A1 promoter in C3H10 (D) and primary chondrocytes (G). Values represent mean ± SD (n = 3). P values were obtained from t-tests with paired or unpaired samples, *P < 0.05, **P < 0.01.

FIGURE 3. Effects of BET inhibitors on Col2a1 expression. A-E and F-J, the effect of BET inhibitors in gradient concentrations was assessed by luciferase activity analysis in ATDC5 cells (A-E) and C3H10 cells (F-J). K-O and P-T, the Col2a1 expression in gradient concentrations of BET inhibitors treatment in ATDC5 (K-O) and primary chondrocytes (P-T). The reported values of 0.1, 1 and 5 are indicated as 0.1, 1 and 5 µM, respectively. Values represent mean ± SD (n = 3). P values were obtained from t-tests with paired or unpaired samples, *P < 0.05, **P < 0.01.

FIGURE 4. I-BET151 and (+)-JQ1 affected the expression of Col2a1, Adamts5 and Mmp3 in chondrocytes. A-F, the expression level of chondrocyte specific marker genes, Col2a1, Adamts5 and Mmp3 as analyzed by quantitative RT-PCR using RNA isolated from cells treated as indicated. The values 0.2, 0.5, 1 and 5 were reported as 0.2, 0.5, 1 and 5 µM, respectively. Values represent mean ± SD (n = 3). P values were obtained from t tests with paired or unpaired samples, *P < 0.05, **P < 0.01. G and H, indicated protein level was assessed by western blot after treatment of chondrocytes with I-BET151 and (+)-JQ1.

FIGURE 5. BET inhibitors repressed chondrocyte differentiation. A, E and F, micromass culture of chondrocytes derived from cartilage articularis of 4-day-old mice (A), C3H10 (E) and ATDC5 (F). The cultures were treated with I-BET151 and (+)-JQ1 at 5 µM and 1 µM respectively for 7 days, and stained using alcian blue. B-D, quantitative RT-PCR analysis was performed for the indicated genes in RNA isolated from micromass cultures of primary chondrocytes (A). G, articular cartilage from distal femurs of newborn mice were treated with I-BET151 at 5 µM and vehicle alone (DMSO) for one week. Top, the comparison of cartilage in DMSO and I-BET151 treatment. Bottom, cartilage-like particle was sectioned and stained with safranin O. H, articular cartilage from distal femurs of newborn mice were treated with (+)-JQ1 at 0.5 µM and vehicle alone (DMSO) for one week. Top, the comparison of cartilage in DMSO and (+)-JQ1 treatment. Bottom, cartilage-like particle was sectioned and stained with safranin O. I and J, a comparison of cartilage volume in DMSO and I-BET151 (I) or (+)-JQ1 treatment (J). Values represent mean ± SD (n = 3). P values were obtained from t tests with paired or unpaired samples, *P < 0.05, **P < 0.01.

FIGURE 6. BET inhibitors removed the binding of Pol II to the promoter regions of the Col2a1. A, POL II protein level was assessed by western blot after treatment of chondrocytes with I-BET151 and (+)-JQ1. B, schematic representation of the mouse Col2a1 gene locus. Positive ChIP–PCR amplification was obtained using primer 1–4. TSS, transcription start site. C, ChIP–qPCR analysis of the distinct
regions in Col2a1 promoter showing enrichment with Pol II antibody compared with IgG controls. Values represent mean ± SD \((n=3)\). \(P\) values were obtained from \(t\)-tests with paired or unpaired samples, **\(P < 0.01\).

**FIGURE 7. BET inhibitors regulated zebrafish skeletal development.** A, schematic diagram of Col2a1 promoter EGFP construct. B, the remarkable EGFP fluorescence signal was detected at 2 dpf. C and D, EGFP fluorescence images of transgenic zebrafish embryos containing EGFP initiated by Col2a1 promoter that treated indicated at 2 dpf. E and H, wild-type zebrafish were continuously treated as indicated for two weeks. F and I, body length of wild-type zebrafish continuously treated as indicated for two weeks. G and J, survival percentage of zebrafish undergoing the indicated treatment. K-N, alizarin red and alcian blue staining of 3-wk-old zebrafish. M and N were the high magnification of K and L respectively. O and P, statistics analysis of the length of centra 15 (c15) under different treatment. Bars = 1 mm in E and H, 2 mm in K and L, and 500 μm in M and N. Values represent mean ± SD \((n=3)\). \(P\) values were obtained from \(t\)-tests with paired or unpaired samples, *\(P < 0.05\), **\(P < 0.01\).

**FIGURE 8. The expression of marker genes in zebrafish.** A-J, analysis of the transcript levels of indicated genes by quantitative PCR using RNA isolated from the wild-type zebrafish which were continuously treated with I-BET151 (1 μM), (+)-JQ1 (0.5 μM) and DMSO as control for three weeks. Values represent mean ± SD \((n=3)\). \(P\) values were obtained from \(t\) tests with paired or unpaired samples, *\(P < 0.05\), **\(P < 0.01\). K, immunohistochemistry analysis of COL2A1 in paraffin sections of DMSO, I-BET151 and (+)-JQ1 treated fish. Bars = 100 μm.
Figure 1

A

\[ \text{Col2a1 promoter (3 kb)} \rightarrow \text{Luciferase} \]

B

C

GFP

\[ \begin{align*}
0 & \quad 0.0005 \\
0 & \quad 0.001 \\
0 & \quad 0.0015 \\
0 & \quad 0.002
\end{align*} \]

\[ \begin{align*}
\text{Relative Luciferase Units} \quad & \text{DMSO} \quad 0.1 \quad 1 \quad 10 \\
\text{KGN} & \text{SOX9}^* \quad ** \quad \text{HA-SOX9}
\end{align*} \]
Figure 2

A. Relative luciferase units for COL2A1-luc reporter with different BET inhibitors.

B. Relative luciferase units for EF1α-Luc reporter.

C. Expression level of Col2a1 with BET inhibitors.

D. Relative luciferase units for COL2A1-luc reporter.

E. Relative luciferase units for EF1α-Luc reporter.

F. Expression level of Col2a1 with BET inhibitors.

G. Relative luciferase units for COL2A1-luc reporter.

H. Relative luciferase units for EF1α-Luc reporter.

I. Expression level of Col2a1 with BET inhibitors.
**Figure 4**

**A**

Col2a1 expression level/β-Actin with I-BET151 treatment.

**B**

Adamts5 expression level/β-Actin with I-BET151 treatment.

**C**

Mmp3 expression level/β-Actin with I-BET151 treatment.

**D**

Col2a1 expression level/β-Actin with (+)-JQ1 treatment.

**E**

Adamts5 expression level/β-Actin with (+)-JQ1 treatment.

**F**

Mmp3 expression level/β-Actin with (+)-JQ1 treatment.

**G**

Western blot analysis showing the expression levels of I-BET151, COL2A1, ADAMTS5, MMP3, Histone H3, and TUBULIN with I-BET151 treatment.

**H**

Western blot analysis showing the expression levels of COL2A1, ADAMTS5, MMP3, Histone H3, and TUBULIN with (+)-JQ1 treatment.

BET Inhibitors Induce Cartilage Destruction
Figure 6

A

|       | DMSO | I-BET151 | (+)-JQ1 |
|-------|------|----------|---------|
| kDa   |      |          |         |
| 180   |      |          |         |
| 65    |      |          |         |
| 45    |      |          |         |

B

-2187 -2032 -1078 -951 -678 -514 -140 +43

TSS

Primer 1 Primer 2 Primer 3 Primer 4

C

Fast enrichment

0 5 10 15 20 25

IgG Pol II IgG Pol II IgG Pol II IgG Pol II

Primer 1 Primer 2 Primer 3 Primer 4

** ** ** **
Table 1. A total of 38 epigenetic compounds.

| Category                        | No  | Product Name | CAS No | Targets                                      |
|---------------------------------|-----|--------------|--------|----------------------------------------------|
| DNA Methyltransferase           | 84  | 5-Aza-2’-deoxycytidine | 7510-67-1 | DNA Methylation                              |
| Epigenetic Reader Domains       | 85  | 5-Aza-2’-deoxycytidine | 7510-67-1 | DNA Methylation                              |
| Histone Methyltransferase       | 86  | 5-Aza-2’-deoxycytidine | 7510-67-1 | DNA Methylation                              |
| Histone demethylases            | 87  | 5-Aza-2’-deoxycytidine | 7510-67-1 | DNA Methylation                              |
| Histone Acetyltransferase       | 88  | 5-Aza-2’-deoxycytidine | 7510-67-1 | DNA Methylation                              |
| HIF                            | 89  | 5-Aza-2’-deoxycytidine | 7510-67-1 | DNA Methylation                              |

Table 1 includes a total of 38 epigenetic compounds.
Bromodomain and Extra-terminal Proteins (BET) Inhibitors Suppress Chondrocyte Differentiation and Restrain Bone Growth
Ningning Niu, Rui Shao, Guang Yan and Weigu Zou

J. Biol. Chem. published online November 7, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.749697

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2016/11/07/jbc.M116.749697.full.html#ref-list-1