BRD4 promotes heterotopic ossification through upregulation of LncRNA MANCR

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Aims
Acquired heterotopic ossification (HO) is a debilitating disease characterized by abnormal extraskeletal bone formation within soft-tissues after injury. The exact pathogenesis of HO remains unknown. It was reported that BRD4 may contribute to osteoblastic differentiation. The current study aims to determine the role of BRD4 in the pathogenesis of HO and whether it could be a potential target for HO therapy.

Methods
Achilles tendon puncture (ATP) mouse model was performed on ten-week-old male C57BL/6J mice. One week after ATP procedure, the mice were given different treatments (e.g. JQ1, shMancr). Achilles tendon samples were collected five weeks after treatment for RNA-seq and real-time quantitative polymerase chain reaction (RT-qPCR) analysis; the legs were removed for micro-CT imaging and subsequent histology. Human bone marrow mesenchymal stem cells (hBMSCs) were isolated and purified bone marrow collected during surgeries by using density gradient centrifugation. After a series of interventions such as knockdown or overexpressing BRD4, Alizarin red staining, RT-qPCR, and Western Blot (Runx2, alkaline phosphatase (ALP), Osx) were performed on hBMSCs.

Results
Overexpression of BRD4 enhanced while inhibition of Brd4 suppressed the osteogenic differentiation of hBMSCs in vitro. Overexpression of Brd4 increased the expression of mitotically associated long non-coding RNA (Mancr). Downregulation of Mancr suppressed the osteoinductive effect of BRD4. In vivo, inhibition of BRD4 by JQ1 significantly attenuated pathological bone formation in the ATP model (p = 0.001).

Conclusion
BRD4 was found to be upregulated in HO and Brd4-Mancr-Runx2 signalling was involved in the modulation of new bone formation in HO.

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Keywords: Brd4, Mancr, Osteogenesis, JQ1, Heterotopic ossification

Article focus
- This study investigated the significant role of Brd4 in the pathogenesis of heterotopic ossification (HO).
- BRD4 serves as a potential target for therapy of HO.

Key messages
- Inhibition of BRD4 suppresses, while overexpression promotes, osteogenic differentiation in human bone marrow mesenchymal stem cells (hBMSCs).
- BRD4 promotes osteogenic differentiation via mitotically associated long non-coding RNA (Mancr) and inhibition of BRD4-Mancr signalling attenuates heterotopic ossification in vivo.

Strengths and limitations
- To our knowledge, this is the first study to propose that the BRD4-Mancr-Runx2 signal is involved in the modulation of new bone formation in HO. The findings might provide a foundation for further research on the clinical treatment of HO.
- There is still a lack of clinical trials to confirm the therapeutic effect of JQ1 on HO.
**Introduction**

Heterotopic ossification (HO) is a pathological process of extraskeletal bone formation that could occur as a result of trauma, severe burns, neurological injury, and orthopaedic intervention.\(^1\)\(^-\)\(^3\) Currently, no treatment or prophylactic measures can sufficiently prevent this clinically devastating complication. Since pathological bone formation often recurs following surgical resection, therapeutic intervention, including surgical excision, is unsatisfactory. Most patients never regain a complete range of motion due to persistent or recurrent contractures.\(^4\)

The histopathological features of acquired HO have been described and summarized previously. Early lesions are often hypercellular and with little bone matrix, while later lesions have prominent bone formation. Acquired HO can form through both endochondral and intramembranous ossification processes. Previous studies suggest that HO might be attributed to aberrant osteogenic potential in mesenchymal stem cells (MSCs).\(^5\) Multiple interacting pathways have been identified as significant in this process including BMP signalling pathway, mTOR signalling pathway, hypoxia-inducible factors (HIF) pathway, retinoic acid receptor (RAR) signalling pathway, and GNAS signalling pathway.

**BRD4** is a ubiquitously expressed protein of the Bromo and Extra-Terminal (BET) family that regulates transcriptional elongation of various types of genes by recognizing N-acetylation of lysine residues on histone tails.\(^6\) It was reported that the BET proteins interact with positive transcription elongation factor (P-TEFB) and RNA polymerase II (Pol II) to facilitate gene transcription.\(^7\) Linked to transcriptional initiation and super-enhancer formation, **BRD4** may contribute to a variety of biological regulation processes including cell proliferation, osteoblastic differentiation and osteoclastogenesis, angiogenesis, and vascular permeability. Transcription activity of transcription factors, such as Gli1,\(^8\) and transductions of pathway, including PI3K signalling\(^9\) and Hedgehog signalling,\(^10\) have been reportedly inhibited with the pharmacological inhibition of BET proteins. However, whether **BRD4** is involved in the pathogenesis of HO is still unknown.

Long non-coding RNAs (lncRNAs) are RNAs with a length of more than 200 nucleotides and which do not encode proteins.\(^10\) Accumulating evidence shows that lncRNAs play a special role in the osteogenic differentiation of stem cells and induced pluripotent stem cells from different sources, such as embryo, bone marrow, adipose tissue, and periodontal ligament.\(^11\)\(^-\)\(^12\) Mitotically associated lncRNA (Mancr) was initially identified as an upregulated lncRNA, in invasive breast cancer associated with cell proliferation, activity, and genomic stability.\(^13\) However, here we show that IncRNA Mancr was upregulated in **BRD4**-overexpressed hBMScs during osteogenic differentiation by microarray analysis. It is suggested that **BRD4** and Mancr signal pathway may be involved in osteogenic differentiation. The current study aims to determine the role of **BRD4** and Mancr in the pathogenesis of HO and to explore the possible molecular mechanism.

**Methods**

**Mouse achilles tendon puncture model.** For ATP mouse model, ten-week-old C57BL/6 J male mice were anaesthetized by isoflurane. A 27-gauge needle was punctured into the Achilles tendon body from the lateral aspect percutaneously and this process was repeated five times at different parts of Achilles tendon body for each mouse. For sham operation, the needle was punctured through the skin without touching the Achilles tendons.

**Experimental mice treatment and grouping.** The mice were assigned to six groups (n = 10 for each group): a model group (ATP), a sham control (sham) group, a JQ1 group (ATP receiving JQ1 treatment), a dimethyl sulfoxide (DMSO) group (ATP receiving DMSO treatment), a shMancr group (ATP receiving shMancr treatment), and a shNC group (receiving shNC treatment). Mice in the JQ1 group were treated with weekly subcutaneous injections of JQ1 (50 mg/kg, 5 ul) one week after ATP modelling, for three weeks, while the DMSO group received a similar volume of carrier containing 5% DMSO (Figure 1a). Mice in the shMancr group were treated with weekly subcutaneous injections of shMancr (1 nmol), 5 ul) one week after ATP modelling, for three weeks, while the shNC group received a similar volume of shMancr. Both JQ1 and DMSO were diluted by 10% 2-hydroxypropyl-b-cyclodextrin

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Inhibition of Brd4 suppresses osteogenic differentiation in human bone marrow mesenchymal stem cells (hBMSCs). a) Alizarin red staining and quantification of hBMSCs transfected with shNC or shBrd4 under osteogenic induction. b) Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of Runx2, Osx, and alkaline phosphatase (ALP) in hBMSCs transfected with shNC or shBrd4 under osteogenic induction. c) Western blot analysis of Runx2, Osx, and ALP in hBMSCs transfected with shNC or shBrd4 under osteogenic induction. d) Alizarin red staining and quantification of hBMSCs treated with dimethyl sulfoxide (DMSO) or JQ1 under osteogenic induction. e) Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of Runx2, Osx, and ALP in hBMSCs treated with DMSO or JQ1 under osteogenic induction. f) Western blot analysis of Runx2, Osx, and ALP in hBMSCs treated with DMSO or JQ1 under osteogenic induction. Data are presented as means and standard deviations. *p < 0.05, one-way analysis of variance. mRNA, messenger RNA; OS, osteogenic differentiation culture.

Fig. 2

carrier (Sigma-Aldrich, USA). Both shMancr and shNC were diluted by pure water (Gene Pharma, China).

Isolation and culture of hBMSCs. Human BMSCs were isolated and purified bone marrow extracted from ten patients (six males, four females), aged between 30 and 65 years, with limb fracture and hip arthroplasty. When the broken end of fracture was cleaned during operation or reaming of femoral medullary cavity was conducted after interception of femoral head, fresh bone marrow (5 ml to 7 ml) was collected, and placed in a 10 ml centrifugal tube. After isolation and purification using density gradient centrifugation, hBMSCs were suspended in low-glucose DMEM (Thermo Fisher Scientific, USA), supplemented with 10% FBS (Thermo Fisher Scientific) and 1% penicillin/streptomycin, and cultured in humidified incubators at 37°C and 5% CO₂.

Cell treatments. To induce osteogenesis, human and mouse progenitor cells were plated in a six-well plate at a density of 2*10⁵ cells/well and cultured for 24 hours respectively. The cells were then switched to osteogenic medium consisting of α-minimum essential medium supplemented with 10% fetal bovine serum (FBS), 50 µg/ml L-ascorbic acid, 0.1 µM dexamethasone, and 10 mM b-glycerophosphate to induce osteogenesis. The medium was changed every day.

RNA extraction and real-time quantitative PCR. Achilles tendon samples were flash-frozen in liquid nitrogen and stored at -80°C. Samples were homogenized separately in Trizol (Thermo Fisher Scientific). For gene expression analysis, Total RNA was extracted from cells according to the manufacturer’s protocol, and 2 µg of total DNA-free RNA was used to synthesize complementary DNA (cDNA) using the ReverTra Ace qPCR RT Kit (Toyobo, Japan). The reactions were set up in 96-well plates using 1 µl cDNA with Thunderbird SYBR qPCR Mix (Toyobo), to which gene-specific forward and reverse PCR primers were added. RT-qPCR was performed under the following conditions: 95°C for ten minutes, followed by 40 cycles of 95°C for ten seconds and 55°C for 34 seconds. Analysis was performed to detect Brd4, Mancr, Runx2, Osx, and ALP expression, and β-actin was used as an internal control. The primer sequences are listed in Supplementary Table i.

Western blot. After protein extraction, protein concentration was determined with a BCA assay (CWBIO, China). A 10% SDS-PAGE gel was loaded with 20 µg of total protein, and the separated proteins were transferred by electrophoretic transfer to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in TBST (50mM tris-buffered saline, pH 7.6, 150mM NaCl, 0.1% Tween 20) and incubated with the primary antibody overnight at 4°C in 5% non-fat dry milk in TBST. Immunolabelling was detected using enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific). The antibodies used for Western blot were from the following sources: anti-Runx2 antibody (Abcam, UK; 1:1,000), anti-Osx antibody (Cell Signalling Technology, USA;
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The small interfering RNA (siRNA) duplexes were predesigned with the online software (Stealth RNAi Pre-Designed siRNAs) provided by Ambion (Thermo Fisher Scientific) and constructed by GenePharma. The siRNAs were verified to be efficient before all experiments. Cells were plated at a concentration of $1 \times 10^5$ cells/well in 6-well plates and transduced with the siRNA using lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Different amounts of 20 μM siRNA duplexes were mixed with 5 μl/well of transfection reagent and Opti-MEM reduced serum medium (Thermo Fisher Scientific) to total volume of 500 μl and incubated for 20 minutes. The mixture was applied to cells for 16 hours at 37°C in 5% CO2. Lentivirus infection. Recombinant lentiviruses containing full-length human Brd4 (LV-Brd4) and Mancr (LV-Mancr) were obtained from Genechem (China). Recombinant lentiviruses targeting Brd4 (shBrd4), Mancr (shMancr), and the scramble control (shNC) were predesigned by BLOCK-IT RNAi Designer software (Thermo Fisher Scientific) and constructed by Gene Pharma. Cells were plated at a concentration of $1 \times 10^5$ cells/well in six-well plates; after 24 hours, hBMSCs were infected by the diluted viral supernatant with 5 μg/ml Polybrene and incubated for 24 hours at 37°C, after which time the medium containing the virus was replaced with fresh medium. As a result, shBrd4 (1 nmol) infection was carried out by using the above steps, such as in knockdown of Brd4 with shRNA. This was followed by incubation with fresh medium for 24 hours, osteogenic differentiation culture for two weeks, and finally stained with alizarin red. (Figure 1b).

Microarray analysis. Human BMSCs were infected by the diluted viral supernatant with 5 mg/ml Polybrene (LV-Brd4 and EV each have two groups), RNA was extracted 48 hours later. Total RNA was extracted using Trizol reagent kit (Invitrogen) according to the manufacturer’s instructions. Total RNA was amplified and labelled by Low Input Quick Amp WT Labeling Kit (Santa, USA), following the manufacturer’s instructions. Labelled cRNA were purified by RNeasy mini kit (GmBH). Each slide was hybridized with 1.65 μg Cy3-labelled cRNA using Gene Expression Hybridization Kit (Santa) in Hybridization Oven (Santa), according to the manufacturer’s instructions. After 17 hours of hybridization, slides were washed in staining dishes (Thermo Fisher Scientific) with Gene Expression.
Wash Buffer Kit (Santa), following the manufacturer’s instructions. Slides were scanned by Agilent Microarray Scanner (Santa) with default settings, Dye channel: Green, Scan resolution = 3 μm, PMT 100%, 20bit. Data were extracted with Feature Extraction software 10.7 (Santa). Raw data were normalized by Quantile algorithm, GeneSpring Software 11.0 (Santa). Differentially expressed genes (DEGs) were also identified by the edgeR package (version 3.12.1) (R Foundation for Statistical Computing, Austria), with general linear model and a threshold of fold change > 2 and false discovery rate (FDR) < 0.05.

Mineralization analysis. For detecting the mineralization, we used OS to induce hBMSCs for 14 days. Cells were washed three times with PBS and fixed with 70% ethanol for ten minutes. After three washes with distilled water, the cells were stained with a 40 mM alizarin red S (Sigma-Aldrich) solution (pH 4.1) for ten minutes to visualize matrix calcium deposition. The remaining dye was washed three times with distilled water, and the stained cells were photographed. For quantification, the calcium deposits were distilled with 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0), then the extracted stain was transferred to a 96-well plate and the absorbance of the samples was measured at 570 nm using a microplate reader (Tecan, Switzerland).

Micro-CT and histological analyses. All specimens were obtained from mice at six weeks after ATP procedure and fixed with 4% paraformaldehyde. For micro-CT scanning, specimens were fitted in a cylindrical sample holder and scanned using a Scanco ICT40 scanner (Scanco Medical, Switzerland) set to 55 kVp and 70 lA. For visualization, the segmented data were imported and reconstructed as 3D images using MicroCT Ray V3.0 software (Scanco Medical).

For histological analysis, specimens were decalcified in 0.5 M EDTA (Sigma-Aldrich) at 4°C. Paraffin-embedded sections were stained with haematoxylin and eosin (H&E) and Safranin O Fast Green (SOFG) to evaluate general structures and bone formation. Immunohistochemical analysis of the specimens was conducted using specific antibodies.

Statistical analysis. Data obtained from experiments in duplicate or triplicate and repeated at least three times were represented as mean and standard deviation (SD). Differences between two groups were analyzed using two-tailed t-test. Comparisons of multiple groups (Runx2, Osx, ALP) was analyzed via one-way analysis of variance (ANOVA). The exact sample size and the number of independent experiments performed, description of the samples and statistical analyses done are also specified in the figure legends. Statistical significance was accepted at p < 0.05. All graphs were generated using Prism V.7 (GraphPad, USA), and all statistical tests were performed using SPSS V.21 (IBM).

The methods used in this article have adhered to the ARRIVE guidelines.
Results

Brd4 is upregulated in endochondral ossification of the ATP model. In the current study, upregulation of Brd4 was found in an ATP model at six weeks compared to the sham-operated group, as revealed by analysis of RNA-seq (Supplementary Figure a). To verify the increased expression of Brd4, Achilles tendon samples were collected and further analyzed. RT-qPCR analysis showed that Brd4 messenger RNA (mRNA) level was increased at six weeks (Figure 1c). The μCT results confirmed new bone formation at tendon (Figure 1d), indicating that the HO model was successfully established. SOFG staining showed that endochondral ossification was obvious at six weeks after tenotomy ATP. Increased Brd4+ cells were observed at the site of pathological bone formation (Supplementary Figure ba and bb). Collectively, these results indicate that Brd4 is upregulated in pathological bone-forming sites of the ATP model.

Inhibition of Brd4 suppresses while overexpression Brd4 promotes osteogenic differentiation of hBMSCs. To verify the effect of Brd4 on osteogenic differentiation, hBMSCs were treated with shRNA to knockdown Brd4. Osteogenic differentiation was suppressed in hBMSCs with knockdown of Brd4, as confirmed by analysis of Alizarin red staining (Figure 2a). mRNA and protein level of osteogenic markers including Runx2, Osx, and ALP was found to be suppressed in hBMSCs with knockdown of Brd4 (Figures 2b and 2c). JQ1 is a Brd4-specific antagonist. To further verify the critical role of Brd4 in osteogenic differentiation, hBMSCs were treated with JQ1 or DMSO control under osteogenic differentiation. After 14 days of osteogenic induction, calcium deposits were reduced with JQ1 treatment, as confirmed by analysis of Alizarin red staining (Figure 2d). Consistent with the result of Alizarin red staining, mRNA and protein levels of Runx2, Osx, and ALP were suppressed either with JQ1 treatment or shBrd4 (Figures 2e and 2f). The result of Alizarin red staining showed that overexpression of Brd4 substantially promotes osteogenic differentiation against JQ1 treatment, as confirmed by increased calcium deposits (Supplementary Figure ca). Besides, mRNA and protein levels of Runx2, Osx, and ALP were upregulated by overexpression of Brd4 (Supplementary Figures cb and cc). Collectively, these results suggest that inhibition of Brd4 suppresses osteogenic differentiation in hBMSCs. Brd4 promotes osteogenic differentiation via lncRNA Mancr. To explore the osteogenic effect of Brd4, we conducted a microarray analysis and found that IncRNA Mancr was upregulated in Brd4-overexpressed hBMSCs during osteogenic differentiation (Figure 3a). Overexpression of Brd4 could promote the transcriptional level of Mancr (Figure 3b). However, knockdown of Brd4 leads to a decrease in Mancr transcription level of Mancr (Figure 3c). In keeping with the effect of Brd4 knockdown,

shMancr suppresses the new formation in vivo. a) Micro-CT (μCT) analysis and quantification of new bone in heterotopic ossification (HO) model treated with shNC or shMancr, n = 10. b) Safranin O Fast Green (SOFG) staining of HO model treated with shNC or shMancr. Scale bar = 200 μm (upper panel), scale bar = 80 μm (lower panel). Mancr, mitotically associated long non-coding RNA.
JQ1 also notably inhibited the transcriptional level of Mancr (Figure 3d). To validate whether Brd4 promotes osteogenic differentiation through Mancr signalling, Brd4-overexpressed hBMSCs were treated with Mancr shRNA. Alizarin red staining showed that the osteogenic effect of Brd4 was suppressed with knockdown of Mancr (Figure 3e). After Mancr shRNA treatment, the mRNA and protein levels of Runx2, OSX, and ALP decreased (Supplementary Figure da and db). Overexpression of Mancr further enhanced the osteogenic potential of Brd4-overexpressed hBMSCs, as confirmed by increased calcium deposits (Figure 3f). In keeping with the result of Alizarin red staining, mRNA and protein level of osteogenic markers (Runx2, Osx, and ALP) were upregulated by overexpression of Mancr (Supplementary figures dc and dd). The result of alizarin red staining showed that the osteogenic effect of overexpressed Mancr was attenuated with knockdown of Runx2 (Figure 3g). Additionally, upregulation of mRNA and protein level of Runx2, Osx, and ALP by overexpression of Mancr was suppressed with Runx2 siRNA treatment (Supplementary Figures de and df). Collectively, these results indicate that Brd4 might regulate osteogenic differentiation through Mancr signalling.

**Inhibition of Brd4-Mancr signalling attenuates heterotopic ossification in vivo.** To validate the osteogenic effect of Brd4 during the pathological process of HO, JQ1 – a Brd4 inhibitor – was administered in HO animal model. The result of μCT analysis showed that JQ1 significantly suppressed the pathological bone formation in HO model. New bone volume in HO model was suppressed by JQ1 administration (p = 0.001, two-tailed Student’s t-test) (Figure 4a). SOFG staining showed that pathological bone was significantly attenuated in the JQ1 treatment group compared to the control group. Absence of bone marrow cavity was observed in JQ1 treatment group (Figure 4b). To validate the effect of Mancr during the process of HO, shMancr was administered locally in pathological bone-forming site in HO model. The results showed that shMancr treatment significantly suppressed the pathological bone formation in HO model, as confirmed by μCT analysis (p = 0.001, two-tailed Student’s t-test) (Figure 5a). The result of SOFG staining showed that pathological bone was notably attenuated in shMancr treatment group compared to negative control group (Figure 5b). Collectively, these results suggest that JQ1 could attenuate the pathological bone formation via interfering Brd4-Mancr-Runx2 axis in HO (Figure 6).

**Discussion**

Höring (1908), Jones (1932), and Rothberg (1942) presented some of the earliest descriptions of trauma-induced ossification of the human Achilles tendon. The ATP model was first described in rats by Buck in 1953.
and in 1983, McClure applied the model to mice and found that ectopic bone developed in 60% of animals by five weeks. Previous studies of an ATP model demonstrated mechanistic pathways of bone growth, healing, and complications that may contribute to HO. To date, ATP is a commonly used post-traumatic model due to its straightforwardness and reproducibility. Although the ossification of the Achilles tendon is a rare event that can occur as a consequence of trauma or surgery, ATP animal studies provide the opportunity to better understand the pathophysiology and pathogenesis under traumatic circumstances.

Previous studies suggest that increased osteogenic potential in osteogenic precursor cells plays a critical role in the pathological process of heterotopic ossification. Aberrant expression of multiple osteogenic-associated genes including Runx2, Bmp4, and tumour growth factor beta (TGFβ) were found to induce the development of heterotopic ossification. In the current study, Brd4 was found to be increased during endochondral ossification in a HO model, which indicated that it might be involved in the pathological process of HO.

Brd4 belongs to Bromodomain and Extra-Terminal domain (BET) protein family, which is an important class of ‘histone reading protein’. The BET proteins recognize histone acetylation through binding to ε-N-lysine acetylation motifs of histone. Previous studies showed that Brd4 played a crucial role in regulating gene transcription through epigenetic interactions between bromodomains and acetylated histones during cellular differentiation processes. It is associated with diverse factors that modulate chromatin dynamics and transcription. Paradise et al. reported that Brd4 was robustly expressed in MC3T3 preosteoblasts and recruited to osteoblast-specific genes. However, the exact mechanism of Brd4 regulation of osteogenesis is still unknown. In the current study, we confirmed that Brd4 was critical for osteogenesis of BMSCs. Overexpression of Brd4 by lentiviral vector promoted human BMSC osteogenic differentiation, while downregulation of Brd4 by shRNA inhibited the osteogenic differentiation.

To further explore the downstream molecular pathway of Brd4 regulation of osteogenesis, we conducted a RNA sequencing analysis during the osteogenic process of Brd4-overexpressing BMSCs. Results showed that lncRNA Mancr was upregulated. Mancr (LINC00704) was a novel Brd4-overexpressing BMSCs. Results showed that lncRNA sequencing analysis during the osteogenic process of Brd4 was critical for osteogenic differentiation. Therefore, we hypothesized that targeting Brd4 through JQ1 treatment would inhibit HO formation. Here we report data to support and confirm that Brd4 protein inhibition could be a promising treatment for HO, as new bone formation was attenuated with JQ1 administration in HO model. In addition, shMancr was injected locally into pathological bone-forming site, which also inhibited HO. These results indicate the application potential of Brd4 for cellular and gene therapy of HO, with further safety and effectiveness studies.

In summary, Brd4 was found to be upregulated in HO and Brd4-Mancr-Runx2 signalling was involved in the modulation of new bone formation in HO. Brd4 serves as a potential target for therapy of HO.

**Supplementary material**

Table showing primers for real-time quantitative polymerase chain reaction analysis of gene expression conducted in this study. Figures showing increased expression of Brd4 in heterotopic ossification model. An ARRIVE checklist is also included to show that the ARRIVE guidelines were adhered to in this study.

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