

RRP12 knockdown inhibited inflammation and p53/NF- κ B pathway and alleviated heterotopic ossification in mice

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ABSTRACT

Heterotopic ossification (HO) is a pathological process, featured by the aberrant mature bone tissue in soft tissues outside the normal skeletal system, commonly occurring following trauma or major joint surgery. Ribosomal RNA Processing 12 Homolog (RRP12) has been confirmed to display multiple roles in ribosome synthesis, DNA damage, cell cycle and cell differentiation processes. However, the regulatory influences of RRP12 in HO progression keep vague and require further investigation. In this project, it was illustrated that RRP12 expression was markedly up-regulated in HO mice. Inhibition of RRP12 retarded the progression of HO in mice. Furthermore, RRP12 knockdown alleviated inflammation. Besides, it was revealed that suppression of RRP12 inactivated the p53/NF- κ B pathway. Lastly, in bone marrow mesenchymal stem cells (BMSCs), it was verified that knockdown of RRP12 suppressed osteogenic differentiation of BMSCs. In conclusion, RRP12 knockdown inhibited inflammation and p53/NF- κ B pathway and alleviated HO in mice. This study not only expands the current understanding of the molecular underpinnings of HO but also identifies RRP12 as a potential actionable target for therapeutic intervention. Nonetheless, further investigations are warranted to delineate RRP12's upstream regulators and evaluate its translational feasibility in clinical settings.

Key words: RRP12; inflammation; heterotopic ossification; p53/NF- κ B pathway.

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Introduction

Heterotopic ossification (HO) refers to one pathological process, in which mature bone tissue abnormally deposits in soft tissues outside the normal skeletal system.^{1,2} This disease often occurs after trauma, spinal cord injury, burns or major joint surgeries.³ Clinically, HO can generate pain around the joints, compression of blood vessels, and limitation of movement, sorely influencing the recovery process and life quality for patients.⁴ The non-steroidal anti-inflammatory drugs and surgery have been applied for preventing and treating HO,⁵ but lacking therapies on specifically targeting the molecular pathogenesis of this disease. Therefore, further investigations on the pathogenesis of HO and exploitations on new treatment methods hold significant clinical importance. In recent years, high-throughput proteomics technology has been verified to be a powerful tool for the discovery of novel biomarkers and potential therapeutic targets for HO.⁶ A serum proteomics study based on iTRAQ firstly discovered that RRP12-like proteins can be the candidate in HO-positive patients.⁷ RRP12 mainly participates in the processing of the precursor rRNA of the 40S small subunit and the nuclear-cytoplasmic transport.⁸ Besides, RRP12 also plays multiple roles in the DNA damage response, cell cycle, and cell differentiation processes.^{9,10} RRP12, as one key regulatory factor for ribosome biosynthesis and cell proliferation, the expression change of it may reflect the metabolic requirements for fibroblast transformation into chondrocytes and subsequent endochondral ossification in HO microenvironment.¹¹ RRP12 is closely associated with cerebral calcification, which may take part in a wide range of clinical manifestations.¹² Therefore, we speculate that RRP12 can exhibit certain roles in HO progression. Despite extensive investigations into the roles of Wnt/ β -catenin, BMP, and Hedgehog pathways in the pathogenesis of HO,¹³ no single pathway has been established to fully account for the complex molecular mechanisms underlying ectopic bone formation. In this context, the p53 and nuclear factor kappa-B (NF- κ B) signaling pathways have emerged as compelling candidates for investigation. The rationale for focusing on this signaling axis is threefold. First, RRP12 has been demonstrated to function as a crucial regulator of p53 activity.¹⁴ Second, NF- κ B signaling is recognized as an essential driver of HO, actively participating in the progression of HO.¹⁵ Third, a clear synergistic regulatory relationship between p53 and NF- κ B.^{16,17} Therefore, the p53/NF- κ B axis may serve as a rational and significant downstream pathway to explore in the context of RRP12-mediated effects on HO.

In the present study, our findings indicated that RRP12 knockdown inhibited inflammation and p53/NF- κ B pathway and alleviated HO in mice. This project suggested that RRP12 is a prospective biomarker for HO development.

Materials and Methods

Mice model

The animal experiments were approved by Ethical approval was obtained from the Ethics Committee of Lu'an people's Hospital (Approval no. 2025LL017). All methods were carried out in accordance with ARRIVE guidelines. The male C57BL/6J mice (12 weeks, n=24) purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were separated into four groups (n=6 mice/group): i) Control group: the needle was punctured through the skin without touching the Achilles tendons; ii) HO group: A 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, generating trauma; iii) HO+shNC group: HO mice were injected with adenovirus expressing shNC (AAV-shNC) into caudal vein; iv) HO+shRRP12 group: HO mice were injected with adenovirus expressing shRRP12 (AAV-shRRP12) into caudal vein.

The HO model was established by percutaneous Achilles tendon puncture using a 27-gauge needle, which was inserted from the lateral approach into the tendon midsubstance, passed through the full tendon thickness, and repeated at five distinct sites. For *in vivo* knockdown of RRP12, AAV-shRRP12 was administered *via* a single tail-vein injection at a dose of 1×10^{11} vg per mouse in 100 μ L sterile PBS at 1 week post-injury.

Western blot

Proteins from the Achilles tendon tissues were separated through RIPA Lysis Buffer (Beyotime, Shanghai, China). The separation of proteins was done through SDS-PAGE (10%), next moving to PVDF membranes. Co-culturing with anti-RRP12 (A304-583A-T; 1/1000; Bethyl Laboratories, Inc., Montgomery, TX, USA), anti-Runx2 (ab192256; 1/1000, Abcam, Shanghai, China), anti-Osx (ab209484; 1/250, Abcam), anti-ALP (ab317130; 1/1000, Abcam, Shanghai, China), anti-p-p65 (ab76302; 1/1000, Abcam), anti-p65 (ab32536; 1/1000, Abcam), anti-p53 (ab131442; 1/1000, Abcam), or anti-GAPDH (ab181602; 1/5000, Abcam, Shanghai, China). The HRP-IgG secondary antibody (ab6721; 1/2000, Abcam, Shanghai, China) were further utilized. The blots were determined through the enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Immunohistochemistry assay

The paraffin-embedded the Achilles tendon tissues (4 μ m) were treated with dewaxing and re-hydration. Post sealing, RRP12 antibody (1/1000, Bethyl Laboratories, Inc.) were mixed for 12 h incubation at 4°C, following secondary antibody (1:1000, ab7090, Abcam). Afterwards, staining (diaminobenzidine) and re-staining (hematoxylin) for the sections were done. Images were got by the light microscope (Olympus Corporation, Tokyo, Japan).

Hematoxylin and Eosin staining

The Achilles tendon tissues were fixed (4% paraformaldehyde), following paraffin embedding. Next, tissues were sliced into 4- μ m-thick sections. Staining with hematoxylin/eosin solution, the tissues were observed by the light microscope (Olympus Corporation).

Masson staining

The paraffin embedding of the Achilles tendon tissues in mice were sliced the sections (4- μ m-thick). The sections were stained by Masson's trichrome (Sigma-Aldrich, St Louis, MO, USA). The images were observed by the light microscope (Olympus Corporation).

RT-qPCR

The TRIzol reagent (Invitrogen) was adopted to isolate RNAs from the Achilles tendon tissues. The reversing RNAs to cDNAs was made by the PrimeScript® RT reagent kit (Takara, Kusatsu, Japan). RT-qPCR was done through the SYBR® Premix Ex Taq™ II kit (Takara). The relative mRNA expression was got through the 2^{- $\Delta\Delta$ Ct} method.

IL-6 forward, 5'-ACTCACCTCTTCAGAACGAATTG-3', and reverse, 5'-CCATCTTTGGAAGGTTTCAGGTTG-3';

IL-1 β forward, 5'-GGATATGGAGCAACAACCTGG-3', and

reverse, 5'-ATGTACCAGTTGGGGAAGT-3';

TNF- α forward, 5'-CTGCACTTTGGAGTGATCGG-3', and reverse, 5'-GCTTGAGGGTTTGCTACAACAT-3';

GAPDH forward, 5'-AACGTGTCAGTGGTGGACCTG-3', and reverse, 5'-AGTGGGTGTCGCTGTTGAAGT-3'.

ELISA

The IL-6 (ab222503; Abcam), IL-1 β (ab197742; Abcam), TNF- α (ab208348; Abcam) kits were employed for evaluation.

Cell line and transfection

Bone marrow mesenchymal stem cells (BMSCs, purchased from StemCell Technologies, Inc., Vancouver, Canada) were cultured with DMEM/F12 medium (10% FBS) at 37°C in one incubator. Cells were confirmed by short tandem repeat (STR). Cells were routinely tested negative for mycoplasma contamination. The shRNAs target RRP12 (sh-RRP12) with negative control (sh-NC) were acquired from GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) was adopted for transfecting these plasmids.

Alizarin red S staining assay

After washing, BMSCs were fixed (4% paraformaldehyde). Subsequently, 1% alizarin red S solution was utilized for staining cells for 30 min. After washing and air-drying, cells were photographed by the light microscope (Olympus Corporation).

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity was tested by using BCIP/NBT Alkaline Phosphatase Color Development Kit and Alkaline Phosphatase Assay Kit. As the standard, protein concentrations were determined by the BCA method. Absorbance at 405 or 562 nm was assessed for ALP activity or protein quantification.

Statistical analysis

The statistical analysis was made through utilizing GraphPad Prism software 9.0 (GraphPad Software, La Jolla, CA, USA). Data were presented as mean $\pm$ SD. The Student's *t*-test or one-way ANOVA by Tukey's test was adopted to analyze the statistical difference. The *p*-value <0.05 is regarded as statistically significant.

Results

RRP12 expression was elevated in HO mice

The protein expression of RRP12 was elevated in HO mice (Figure 1A). Besides, the same change of RRP12 was confirmed through IHC assay (Figure 1B). Taken together, these data suggested that RRP12 expression was elevated in HO mice.

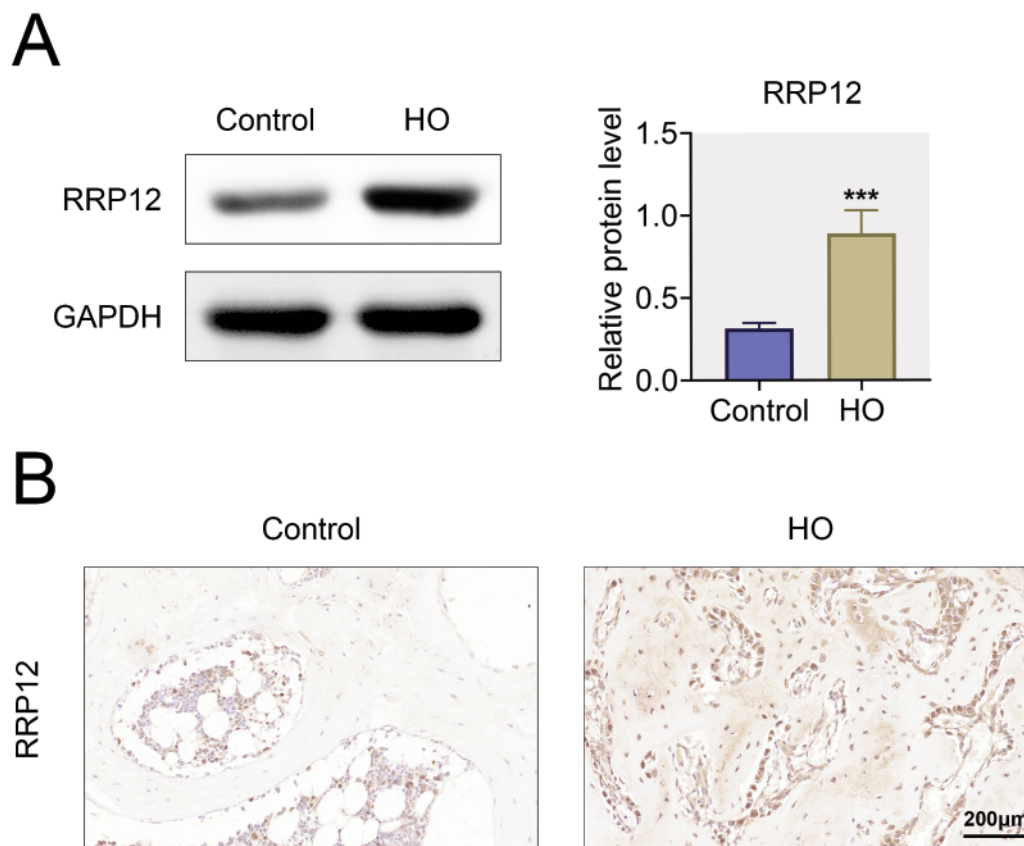


Figure 1. RRP12 expression was elevated in HO mice. **A)** Protein expression of RRP12 in the Control and HO groups was examined through Western blot. **B)** Protein expression of RRP12 in the Control and HO groups was evaluated through IHC assay. ****p*<0.001.

Inhibition of RRP12 retarded the progression of HO in mice

The protein expressions of RRP12, Runx2, Osx and ALP were up-regulated in HO mice, but these impacts were reversed after RRP12 knockdown (Figure 2A). The inflammatory cell infiltration was strengthened in HO mice, but this change was offset after RRP12 inhibition (Figure 2B). In addition, the excessive deposition of collagen had happened in HO mice, but this phenomenon was attenuated after RRP12 reduction (Figure 2C). In a word, inhibition of RRP12 retarded the progression of HO in mice.

RRP12 knockdown alleviated inflammation

The mRNA expressions of IL-6, IL-1 β and TNF- α were aggrandized in HO mice, but these influences were counteracted after RRP12 inhibition (Figure 3A). Moreover, through ELISA, it was demonstrated that the IL-6, IL-1 β and TNF- α levels were lifted in HO mice, but these effects were rescued after RRP12 knock-

down (Figure 3B). In general, RRP12 knockdown alleviated inflammation.

Suppression of RRP12 inactivated the p53/NF- κ B pathway

The protein levels of p-p65/p65 and p53 were increased in HO mice, but these impacts were attenuated after RRP12 suppression (Figure 4), indicating that suppression of RRP12 inactivated the p53/NF- κ B pathway.

Knockdown of RRP12 inhibited osteogenic differentiation of BMSCs

In BMSCs, the protein expressions of RRP12, Runx2, Osx and ALP were declined after RRP12 inhibition (Figure 5A). Additionally, mineralization level was relieved after RRP12 down-regulation (Figure 5B). The ALP activity was weakened after RRP12 knockdown (Figure 5C). In short, knockdown of RRP12 inhibited osteogenic differentiation of BMSCs.

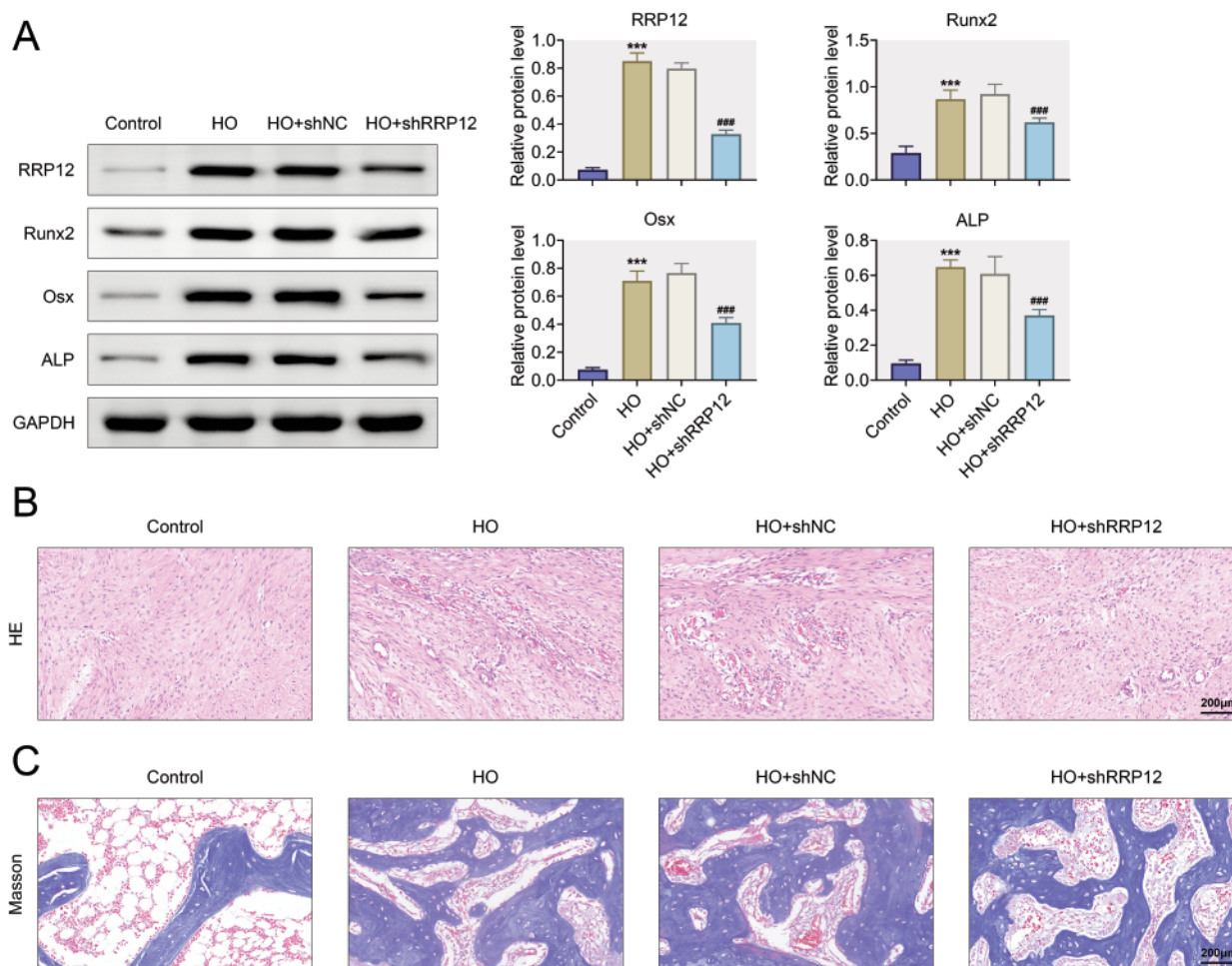


Figure 2. Inhibition of RRP12 retarded the progression of HO in mice. Groups were separated into the Control, HO, HO+shNC and HO+shRRP12 groups. **A)** Protein expressions of RRP12, Runx2, Osx and ALP were assessed through Western blot. **B)** Changes in tissues were observed through H&E staining. **C)** Pathological changes were examined through Masson staining. *** p <0.001 vs Control group; ### p <0.001 vs HO+shNC group.

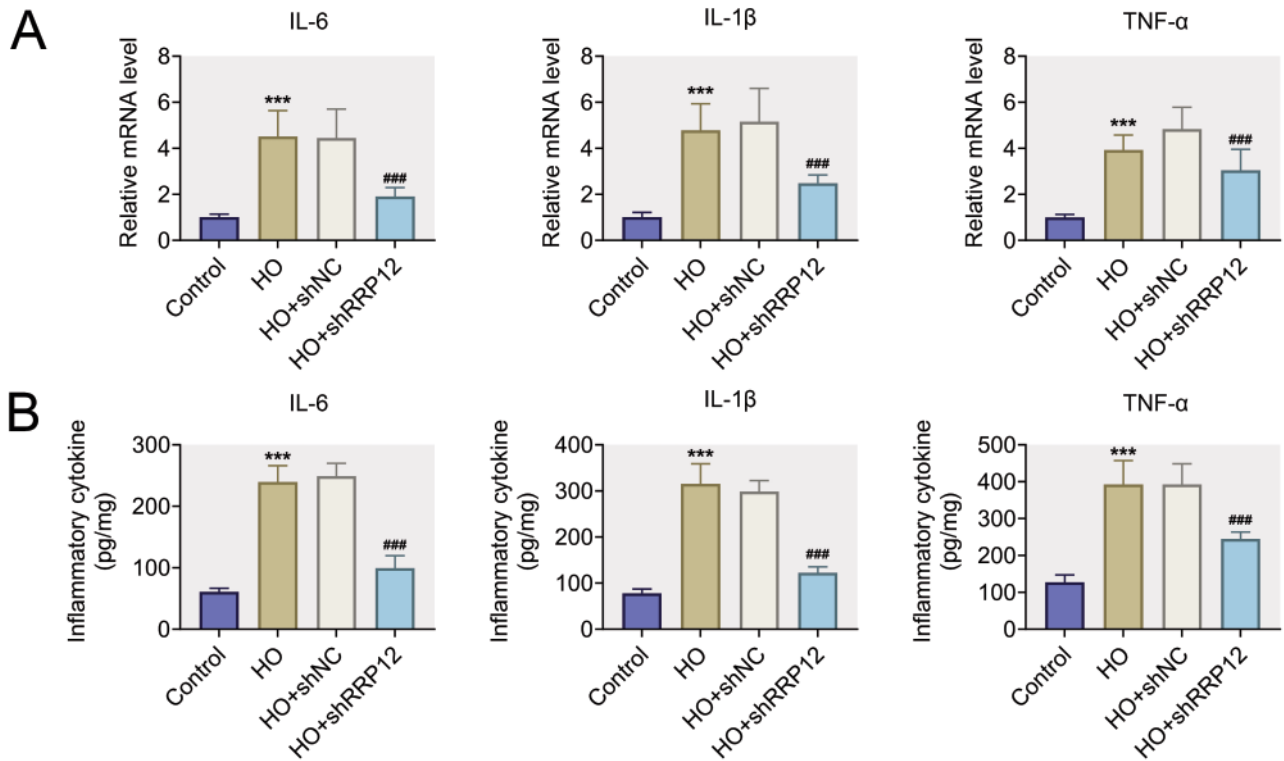


Figure 3. RRP12 knockdown suppressed inflammation. Groups were separated into the Control, HO, HO+shNC and HO+shRRP12 groups. **A)** mRNA expressions of IL-6, IL-1 β and TNF- α were detected through RT-qPCR. **B)** Levels of IL-6, IL-1 β and TNF- α were assessed through ELISA. *** p <0.001 vs Control group; ### p <0.001 vs HO+shNC group.

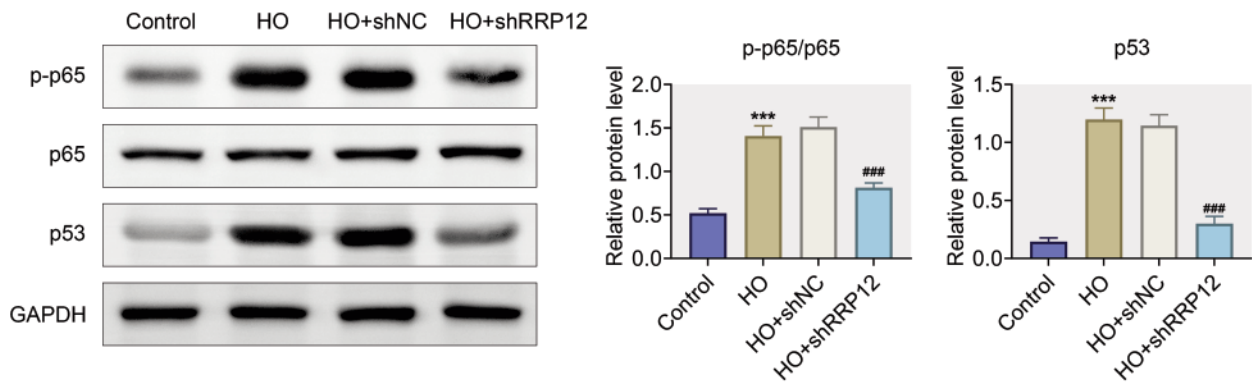


Figure 4. Suppression of RRP12 inactivated the p53/NF- κ B pathway. Groups were separated into the Control, HO, HO+shNC and HO+shRRP12 groups; Protein expressions of p-p65, p65 and p53 were determined through Western blot. *** p <0.001 vs Control group; ### p <0.001 vs HO+shNC group.

Discussion

RRP12 has been notarized to exhibit multiple roles in ribosome synthesis, cell cycle and cell differentiation processes.^{9,10} However, the regulatory influences of RRP12 in HO progression remain unclear and require further explorations. In this work, it was discovered that RRP12 expression was elevated in HO mice. Inhibition of RRP12 retarded the progression of HO in mice.

Inflammation is a vital progress in HO.^{18,19} Many reports have focused on the regulation of inflammation in HO development. For example, the IL-33/ST2 Axis can aggravate inflammation, thereby accelerating traumatic HO progression.²⁰ In addition, PRMT6 increases CCL2 expression to strengthen macrophage-evoked inflammation in HO.²¹ Quercetin modulates immune cell infiltration and inflammation to improve trauma-triggered HO.²² Furthermore, suppression of focal adhesion kinase 2 can regulate macrophage polarization to relieve inflammation, thereby ameliorating HO progression.²³ Clinical studies have also shown that although anti-inflammatory treatment cannot completely prevent the bone generation, it can reduce the happening of HO after major hip surgeries.^{18,24} In this work, it was found that RRP12 knockdown can alleviate inflammation.

It has been affirmed that in osteosarcoma, RRP12 can influence the p53 activity.¹⁴ Interestingly, inactivation of NF- κ B retarded the progression of trauma-evoked HO rat model.²⁵ Palovarotene can inhibit the NF- κ B pathway to ameliorate HO.²⁶ Additionally, Ethyl caffeate affects the SIRT1/NF- κ B pathway to suppress macrophage polarization, thereby improving traumatic HO.²⁷ Moreover, metformin retards the NF- κ B pathway to mitigate trauma-triggered HO.²⁸ Importantly, p53 and NF- κ B co-promote

proinflammatory cytokines.²⁹ However, the relationship between RRP12 and the p53/NF- κ B pathway keep unclear. In this project, it was discovered that suppression of RRP12 inactivated the p53/NF- κ B pathway in HO mice. Lastly, in BMSCs, knockdown of RRP12 inhibited osteogenic differentiation.

The pathological progression of HO follows a well-defined temporal sequence: inflammation, chondrogenesis, osteogenesis, and finally bone maturation, with inflammation preceding osteogenic differentiation.^{30,31} RRP12 may act as an upstream nodal regulator in this cascade, coupling inflammatory signals to the osteogenic differentiation program through modulation of the p53/NF- κ B signaling axis. The observation that RRP12 knockdown concurrently suppresses both inflammation and osteogenesis supports the notion that RRP12 functions at this critical intersection. However, due to the absence of multi-time-point dynamic monitoring in the current experimental design, our data only establish correlative associations and cannot rigorously demonstrate causality or the temporal hierarchy of these events.

In conclusion, these findings elucidated that RRP12 knockdown inhibited inflammation and p53/NF- κ B pathway and alleviated heterotopic ossification in mice. Nevertheless, some limitations (missing explorations on other cellular phenotypes and clinical samples) also happen in this work. A primary limitation of this investigation pertains to the absence of a direct comparative analysis between RRP12-targeted interventions and established clinical pharmacotherapies for heterotopic ossification (HO). Furthermore, the *in vivo* safety profile, tolerability, and translational feasibility of targeting RRP12 remain to be systematically evaluated, thereby constraining the immediate clinical applicability of these findings. To elucidate the mechanistic role and therapeutic potential of

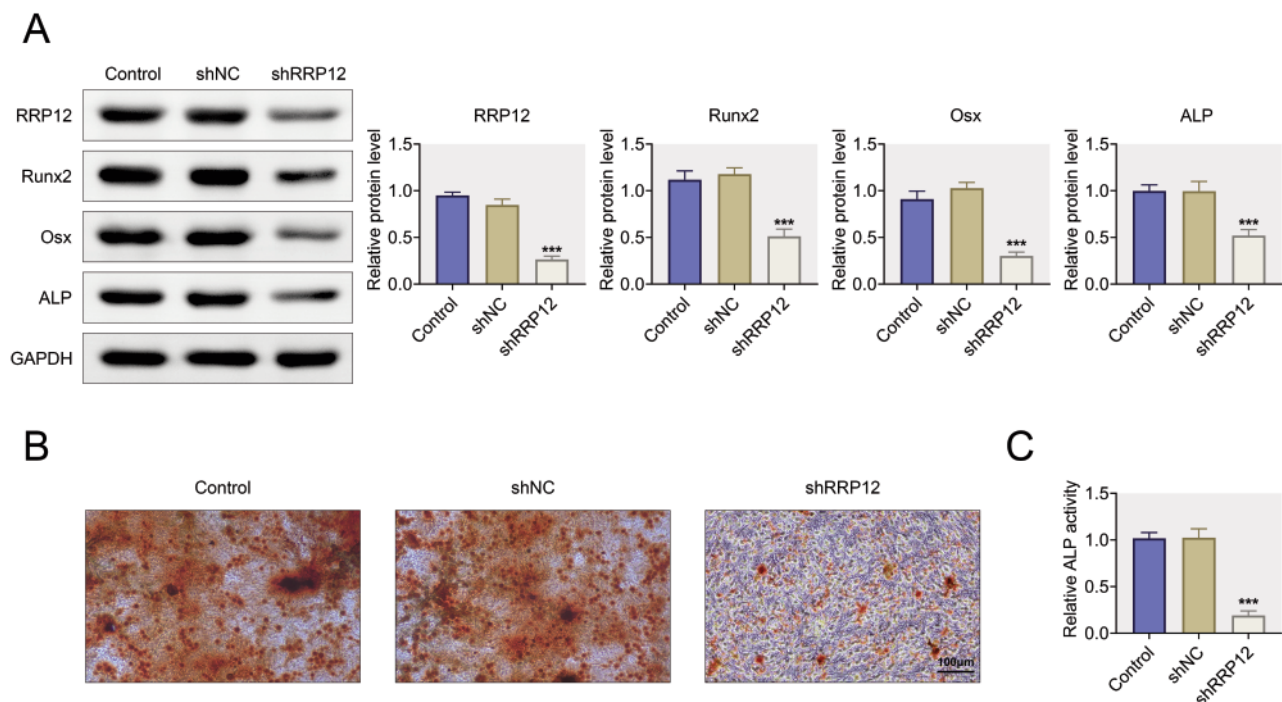


Figure 5. Knockdown of RRP12 inhibited osteogenic differentiation of BMSCs. Groups were separated into the Control, shNC and shRRP12 groups. **A)** Protein expressions of RRP12, Runx2, Osx and ALP were detected through Western blot. **B)** Mineralization level was determined by alizarin red S staining. **C)** ALP activity was confirmed through ALP activity assay. *** $p < 0.001$.

RRP12 in HO pathogenesis, subsequent studies will focus on: i) multi-time-point dynamic monitoring of RRP12 expression, inflammatory cytokine profiles, and osteogenic marker levels to delineate the temporal sequence of these events during HO progression; ii) implementing phase-targeted RRP12 inhibition during distinct pathological stages (specifically differentiating early inflammatory cascades from late-stage osteogenic lineage commitment) to establish optimal intervention windows; and iii) *in vitro* co-culture model systems to evaluate the functional crosstalk and potential causal hierarchy between RRP12-mediated inflammatory signaling and osteogenic differentiation.

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