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The dual role of titanium particles in osteolysis: implications for gene therapy in prosthesis loosening

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Abstract

Background Aseptic prosthesis loosening caused by wear particles is a major complication in patients with osteoarthritis following total joint replacement. Despite advancements in treatment, the underlying mechanisms remain poorly understood, and effective therapies are still lacking.

Methods In this study, we investigated the effects of titanium particles on osteoclast and osteoblast differentiation through both in vitro and in vivo experiments.

Results Our findings revealed that titanium particles not only promote the differentiation of RAW264.7 cells into osteoclasts and enhance the secretion of inflammatory factors but also inhibit the differentiation of BMSCs into osteoblasts and reduce the expression of Wnt signaling pathway-related factors. Furthermore, using a mouse model of knee prosthesis loosening and AAV-mediated gene therapy, we demonstrated that the combination of *TNF-a* interference and *Wnt3a* overexpression was more effective than single-gene therapy in reducing inflammatory cell infiltration, promoting bone formation, and mitigating bone destruction.

Conclusions These results highlight the dual role of titanium particles in periprosthetic osteolysis and underscore the potential of a gene therapy strategy targeting both inflammatory factors and the Wnt signaling pathway to improve knee prosthesis loosening. This study provides a scientific foundation for developing novel therapeutic approaches.

Keywords Aseptic prosthesis loosening, Titanium particles, Osteolysis, Gene therapy, *TNF-a*, *Wnt*3a

Introduction

Osteoarthritis (OA) is one of the most common joint disorders, with an estimated 3 million new cases diagnosed annually [1]. When conservative treatments fail, patients often undergo total joint replacement surgery [2]. However, aseptic loosening, primarily caused by periprosthetic osteolysis, remains a significant long-term complication following total joint arthroplasty [3–5]. Despite progress in wear-resistant materials, surgical techniques, and pharmacological interventions, aseptic loosening continues to pose a major clinical challenge.

Aseptic loosening is typically triggered by an inflammatory response to wear particles, leading to bone resorption and eventual implant instability [6]. Notably,

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wear particles can activate macrophages, resulting in the release of inflammatory factors, such as tumor necrosis factor-alpha (*TNF-α*), which promotes bone resorption and destruction of surrounding bone tissue [7–9]. Research has shown that the size, shape, and chemical composition of wear particles significantly influence their biological reactivity, with smaller particles more likely to be engulfed by cells and elicit stronger inflammatory responses [7]. In addition, metal wear particles, such as those from cobalt–chromium alloys, have been found to induce more intense local inflammatory reactions, exacerbating osteolysis [10]. Furthermore, studies suggest that wear particles indirectly contribute to aseptic loosening by activating bone resorption pathways [11]. Therefore, elucidating the molecular mechanisms by which wear particles influence osteolysis is crucial for developing effective treatment strategies.

Recent studies have highlighted the critical role of wear particles, particularly titanium particles, in the pathogenesis of peri-implant osteolysis [12, 13]. Titanium particles, generated from implant wear, have been shown to can influence the function of bone cells through multiple signaling pathways. For instance, titanium particles can activate the nuclear factor κB (NF-κB) signaling pathway, promoting inflammatory responses and the formation of osteoclasts [13]. Moreover, the presence of titanium particles can induce oxidative stress, further exacerbating bone resorption [14]. Titanium particles also regulate the balance between bone resorption and formation by modulating the expression of key cytokines, such as altering the ratio of RANKL to OPG [15].

Periprosthetic osteolysis results from an imbalance between osteoclastic bone resorption and osteoblastic bone formation [16, 17]. Mesenchymal stem cells (MSCs), known for their multipotency, can differentiate into osteoblasts under the regulation of signaling pathways, such as bone morphogenetic protein (BMP), Wnt, and Notch. These pathways have shown therapeutic potential in cartilage repair and osteoarthritis management [18–21]. Studies have demonstrated that wear particles can inhibit the Wnt signalling pathway, thereby impairing osteoblast differentiation and function [22]. However, a systematic analysis of the effects of titanium particles on osteoclast differentiation and osteoblast formation is still lacking.

In this study, we aimed to address two key questions. First, we sought to investigate the dual role of titanium particles in osteoclast and osteoblast differentiation, with a focus on their effects on osteolysis. Second, we aimed to explore the potential of gene therapy targeting *TNF-α* and *Wnt3a* in mitigating prosthesis loosening, utilizing AAV-mediated gene delivery. By addressing these objectives, we aim to provide a comprehensive understanding of the mechanisms underlying titanium particle-induced osteolysis and to develop novel therapeutic strategies to improve the outcomes of joint replacement surgeries.

Methods

Titanium particle preparation

Titanium particles (Ti) with an average diameter of 5 μm were used to induce osteolysis. To eliminate endotoxin contamination, the particles were subjected to a series of treatments. First, they were calcined at 180 °C for 45 min, followed by immersion in concentrated hydrochloric acid for 6 h. After centrifugation to remove the acid, the particles were rinsed in 75% ethanol for 48 h. Endotoxin levels were carefully monitored and confirmed to be below 0.1 EU/mL using a commercial detection kit (E-Toxate; Sigma, USA). A titanium suspension was prepared by dispersing 10 mg of titanium particles in sterile phosphate-buffered saline (PBS).

Construction of adeno-associated virus (AAV)

siRNA oligos targeting *TNF-α* were synthesized by Sangon Biotech, annealed, and cloned into pAdEasy-U6-CMV-EGFP vectors. The plasmid pHBHD-U6-*TNF-α*-siRNA-CMV-*Wnt3a* was linearized with PacI and co-transformed with pAdeasy-1 into E. coli BJ5183 for dual expression of *Wnt3a* and *TNF-α*-siRNA. Positive clones were verified by PCR and sequencing. AAV packaging and purification were performed by Hanheng Biological.

Osteoclast differentiation and TRAP staining

Mouse macrophage RAW264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution at 37 °C in a 5% CO_2 atmosphere. Osteoclast differentiation was induced by treating the cells with RANKL (50 ng/mL) for 5 days [19]. To simulate periprosthetic osteolysis, titanium particles (0.1 mg/mL) were added to the culture. Osteoclasts were then stained using a Tartrate-resistant acid phosphatase (TRAP) staining kit (Servicebio, G1492).

Osteoblast differentiation and ALP staining

Mouse mesenchymal stem cells (MSCs) were maintained in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic solution at 37 °C in a 5% CO_2 atmosphere. Osteogenic differentiation was initiated using DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 mM vitamin C. Titanium particles (0.1 mg/mL) were added to the cultures to inhibit osteoblast differentiation. After 21 days of differentiation, cells were harvested for subsequent

experiments. Alkaline phosphatase (ALP) activity was assessed using an ALP staining kit (Servicebio, G1480).

Aseptic loosening model of knee prosthesis

The mouse model of aseptic loosening was established as previously described with minor modifications [23]. Briefly, mice were anesthetized with 10% chloral hydrate (30 mg/kg) administered intraperitoneally. After removing the fur, a 5 mm bone tunnel was created in the tibial plateau using a dental drill with a 0.8 mm burr. According to the experimental design, the mice were randomly divided into four groups: the normal control group (NC group) which was not treated with titanium particles, the positive control group (PC group) which was treated with titanium particles first and then implanted with titanium nails, the monotherapy group with *TNF-α* knock-down and the combination therapy group with *TNF-α* knock-down and *Wnt3a* overexpression.

Total RNA extraction and quantitative real-time PCR (q-PCR)

Total RNA was extracted using TRIzol reagent. First-strand cDNA was synthesized using a reverse transcription kit (Vazyme, R211). q-PCR was performed using SYBR Green PCR Master Mix. Primer sequences are listed in Supplementary Table 1.

Cell proliferation assay

Cell proliferation was evaluated using cell viability and EdU incorporation assays. Briefly, 2×10^4 cells were seeded in 96-well plates. Cell viability was assessed using the CCK-8 assay (Beyotime, C0038). EdU labeling was performed according to the manufacturer's instructions using the EdU Assay Kit (Ribobio, C10310).

Enzyme-linked immunosorbent assay (ELISA)

Cytokine expression levels in cell culture supernatants and murine sera were quantified using ELISA kits specific for IL-1β (Solarbio, SEKM-0002), *TNF-α* (Solarbio, SEKM-0034), and IL-6 (Solarbio, SEKM-0007) following the manufacturer's protocol.

RNA-sequencing (RNA-seq)

RNA was extracted using TRIzol reagent and quantified using a 2100 Bioanalyzer. Libraries were prepared with the KAPA Stranded mRNA-Seq kit (Kapa Biosystems, KK8420) and aligned to the mm9 genome using Hisat2 [24]. Differential gene expression analysis was performed using DESeq2.

Micro-CT analysis

Micro-CT was used to evaluate peri-implant osteolysis around titanium nails. Scan settings included a 15 μm slice thickness, 45 kV voltage, and 435 μA current. A region of interest (ROI) with a diameter of 1.7 mm adjacent to the tibial growth plate was analyzed, and morphometric parameters such as bone mineral density (BMD) and bone volume fraction (BV/TV) were quantified.

AAV-mediated gene therapy

Starting from the second week, mice received intra-articular injections of 40 μL in the operated limbs biweekly. The control group received PBS injections, while the treatment groups were injected with Ad-*TNF-α*-siRNA or Ad-*TNF-α*-siRNA-OE-*Wnt3a*. This regimen was maintained for 16 weeks, during which mice were fed a standard diet.

Histological staining

Periprosthetic tissues were harvested, fixed in paraformaldehyde, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. After deparaffinization and rehydration, sections were stained with hematoxylin and eosin (H&E) using a Servicebio kit. Slides were dehydrated, cleared, mounted with neutral resin, and examined under an Mshot MF53 microscope for histological analysis.

Von kossa staining

Mice were euthanized at 16 weeks, and tibial bones were isolated and fixed in 4% paraformaldehyde at 4 °C overnight. Fixed samples were dehydrated in sucrose solutions (5% to 30%), embedded in OCT, and sectioned at 10 μm. Von Kossa staining (Solarbio, G3282) was performed as follows: sections were washed in distilled water for 30 min, incubated with silver nitrate, exposed to UV light for 30 min, and treated with sodium thiosulfate for 10 min at room temperature before microscopic examination.

Statistical analysis

Statistical analysis and graphing were performed using SPSS 20.0 and Prism 8.0. Experiments were conducted with three biological replicates, and data are presented as mean ± standard deviation. One-way ANOVA with Sidak's post hoc test was used for groups with equal variances, and Dunnett's T3 test was used for groups with unequal variances. A p value < 0.05 was considered statistically significant.

Results

Ti particle promotes RAW264.7 differentiation into osteoclasts and activates inflammatory response

To investigate the role of titanium particles in osteoclast differentiation, TRAP staining was performed. The results revealed a significant increase in TRAP-positive

osteoclasts in the differentiation group compared to the control group, with an even greater increase in the titanium particle-treated group (Fig. 1A). This finding was further confirmed by q-PCR (Fig. 1B). To assess whether titanium particles affect cell proliferation, we performed EdU staining and CCK-8 assays. The results showed that titanium particles did not influence cell proliferation (Supplementary Fig. 1A–C). However, ELISA results demonstrated that titanium particles significantly increased the levels of *TNF-α*, IL-6, and IL-1β in the cell culture supernatant compared to the differentiation-only group (Fig. 1C). These results indicate that titanium particles not only promote osteoclast differentiation but also enhance the secretion of inflammatory factors.

To further elucidate the molecular mechanisms underlying titanium particle-induced osteoclast differentiation, we performed RNA sequencing (RNA-seq). Principal component analysis (PCA) showed that the three replicates of each sample clustered closely, indicating the reliability of the RNA-seq data (Supplementary Fig. 1D). A volcano plot of differentially expressed genes (DEGs) revealed significant differences between the differentiation group and the differentiation group treated with titanium particles (Fig. 1D). Upregulated genes included

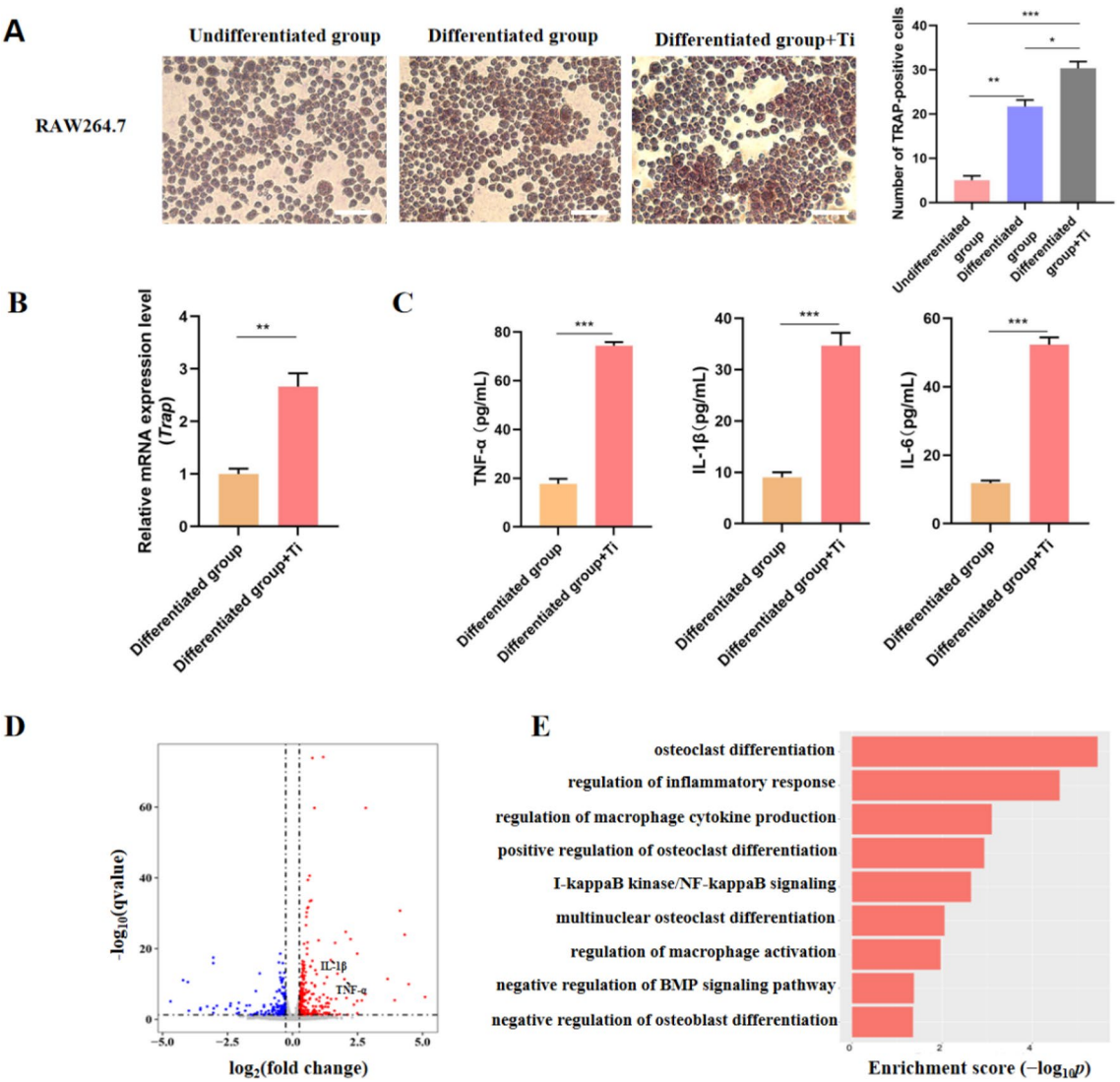

Fig. 1 Ti particle promotes RAW264.7 differentiation into osteoclasts and activates inflammatory response. **A** TRAP staining and quantification in RAW264.7 cells. **B** Trap mRNA levels of RAW264.7 cells. **C** Expression levels of *TNF-α*, IL-1β and IL-6 inflammatory factors in RAW264.7 cells. **D** Volcano map of the differentially expressed mRNA between the two groups. Red: up-regulated differential genes; blue: down-regulated differential genes. **E** GO enrichment analysis of differentially expressed genes.***P < 0.001, **P < 0.01, and *P < 0.05. Scale bar: 40 μm

TNF-α and IL-1β, consistent with the ELISA results (Fig. 1C). KEGG pathway analysis indicated that DEGs were associated with osteoclast differentiation, TNF signaling, and cellular senescence (Supplementary Fig. 1E). Gene Ontology (GO) analysis further revealed that DEGs were related to osteoclast differentiation, NF-κB signaling, and negative regulation of BMP signaling and osteoblast differentiation (Fig. 1E). These findings suggest that titanium particles promote osteoclast differentiation while inhibiting osteogenic gene expression.

Ti particle inhibits BMSC differentiation into osteoblasts and downregulates *Wnt* pathway-related factors

We next investigated the effects of titanium particles on the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). Alizarin red staining showed that the number of positive osteoblasts in the osteoblast differentiation group increased significantly compared to the undifferentiated control group, while the number of positive osteoblasts in the titanium particle-treated group decreased significantly (Fig. 2A). ALP staining further confirmed this observation (Fig. 2B). In addition, qRT-PCR analysis revealed that the expression levels of osteoblast-related genes (Runx2, Ocn, and Opg) were significantly reduced in the titanium particle-treated group compared to the differentiation group (Fig. 2C), indicating that titanium particles inhibit the differentiation of BMSCs into osteoblasts. Similarly, EdU staining and CCK-8 assays showed that titanium particles did not affect cell proliferation (Supplementary Fig. 2A–C).

Subsequent gene expression analysis by RNA-seq revealed significant differences between the differentiation group and the differentiation group treated with titanium particles (Supplementary Fig. 2D). Volcano plot

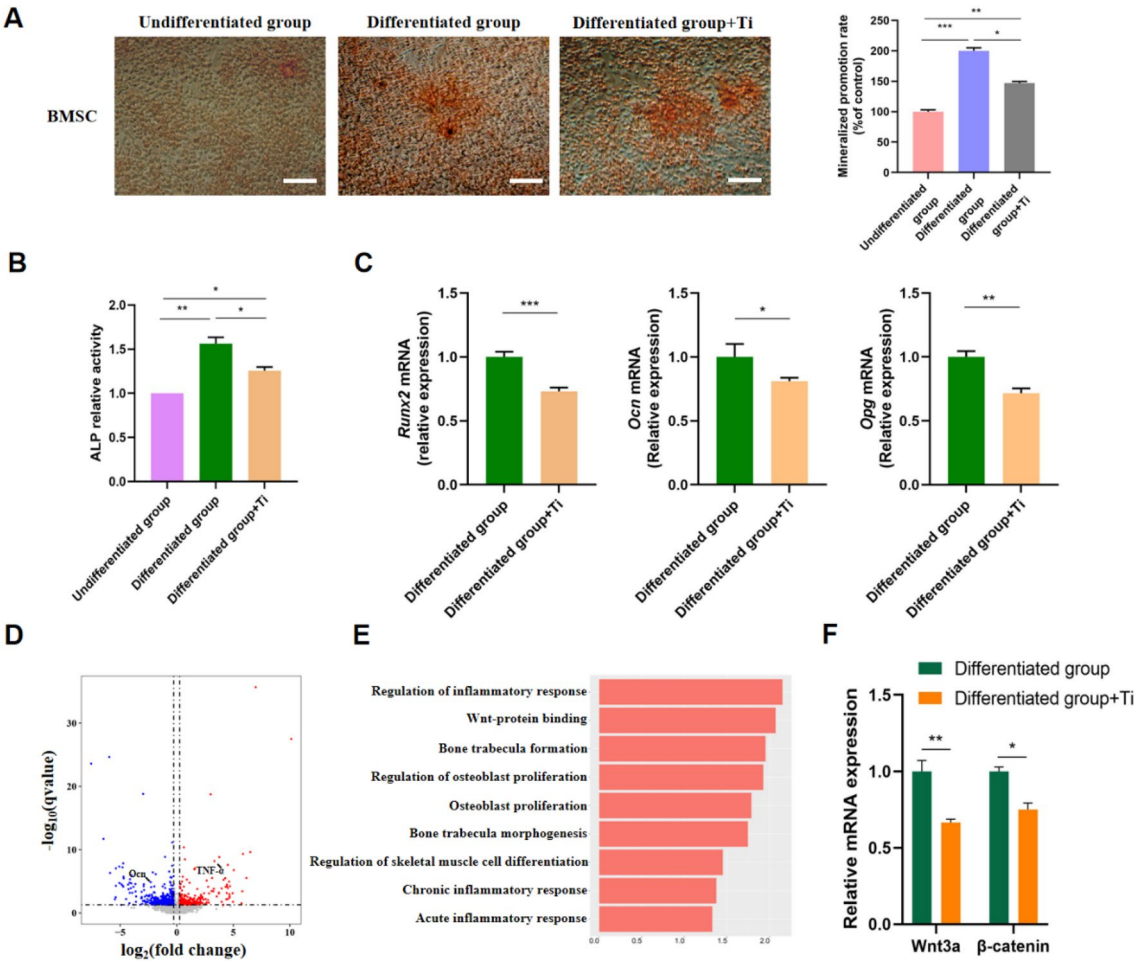

Fig. 2 Ti particle inhibits BMSC differentiation into osteoblasts and downregulates Wnt pathway-related factors. **A** Alizarin red staining and quantification of BMSCs after osteogenic differentiation for 21 days. **B** ALP staining of BMSCs after osteogenic differentiation for 7 days. **C** mRNA expression levels of the osteogenic differentiation markers Runx2, OCN, and OPN after 7 days. **D** Volcano map of the differentially expressed mRNA between the two groups. Red: up-regulated differential genes; Blue: down-regulated differential genes. **E** GO enrichment analysis of differentially expressed genes. **F** mRNA expression levels of *Wnt3a* and β-catenin. ***P < 0.001, **P < 0.01, and *P < 0.05. Scale bar: 40 μm

analysis showed that 221 genes were upregulated and 331 genes were downregulated, with Ocn being enriched among the downregulated genes and *TNF-α* among the upregulated genes (Fig. 2D). KEGG pathway analysis indicated that DEGs were associated with cellular senescence, TNF signaling, and calcium signaling pathways (Supplementary Fig. 2E), consistent with findings in RAW264.7 cells (Supplementary Fig. 1E). GO enrichment analysis revealed that these genes were involved in Wnt-protein binding, bone trabecular formation, and NF-κB signaling (Fig. 2E). Since the binding of Wnt protein to the Frizzled receptor is a key step in activating the Wnt/β-catenin signaling pathway, these results suggest that titanium particles may disrupt this pathway. Further q-PCR analysis confirmed that the expression levels of *Wnt3a* and β-catenin were downregulated in the titanium particle-treated group (Fig. 2F). In summary, these findings demonstrate that titanium particles inhibit osteoblast differentiation and downregulate genes associated with the Wnt signaling pathway.

Construction and validation of the mouse model of knee prosthesis

Given the above findings, we sought to explore the roles of *TNF-α* and *Wnt3a* in the treatment of knee prosthesis loosening (Fig. 3A). Postoperative imaging confirmed the accurate implantation of titanium nails into the knee joint cavity (Fig. 3B and Supplementary Fig. 3B). Two weeks after model establishment, we collected tissues surrounding the knee prosthesis and measured the expression levels of inflammatory factors. q-PCR and ELISA results showed that the expression levels of *TNF-α*, IL-6, and IL-1β were significantly elevated in the PC group compared to the NC group (Fig. 3C, D), indicating that the model successfully replicated the molecular characteristics of aseptic prosthesis loosening.

Comparison of the effectiveness of monotherapy and combination therapy

We developed a targeted gene therapy approach using AAVs and administered intra-articular injections 2 weeks after model establishment (Fig. 4A). After 16 weeks, we euthanized the mice and collected tissue samples for histological analysis. H&E staining revealed significant inflammatory cell infiltration, thick boundary membrane formation, and bone destruction in the PC group (Supplementary Fig. 4A). In contrast, the *TNF-α*-siRNA monotherapy and combination therapy groups showed reduced inflammatory cell infiltration and thinner boundary membranes, with no significant bone destruction. Statistical analysis further indicated that the combination therapy group had fewer inflammatory cells than the monotherapy group (Supplementary Fig. 4A). Consistent with this, *TNF-α* expression was significantly reduced after gene therapy (Supplementary Fig. 4B). In addition, the expression levels of osteoblast-related genes

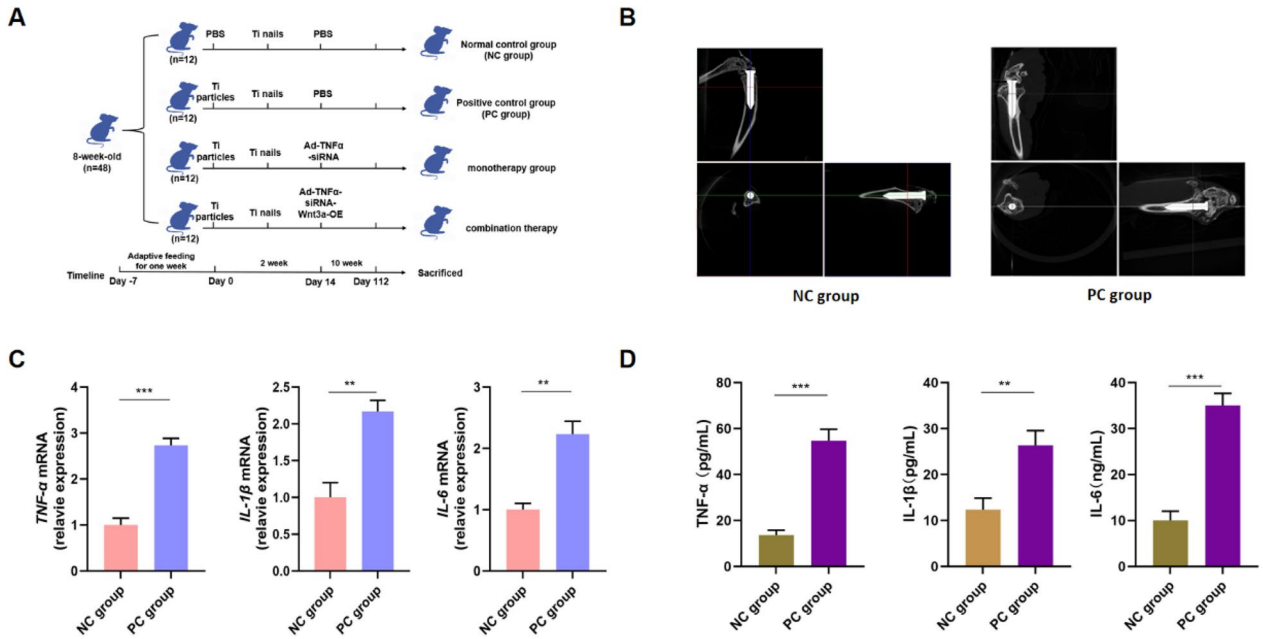

Fig. 3 Construction and validation of the mouse model of knee prosthesis. **A** Animal grouping and flowchart. **B** Postoperative CT scan of mouse knee joint. **C** mRNA relative expression levels of *TNF-α*, IL-1β and IL-6 inflammatory factors in the tissues surrounding mouse prostheses. **D** Expression levels of *TNF-α*, IL-1β and IL-6 inflammatory factors in the tissues surrounding mouse prostheses. ***P < 0.001, **P < 0.01, and *P < 0.05

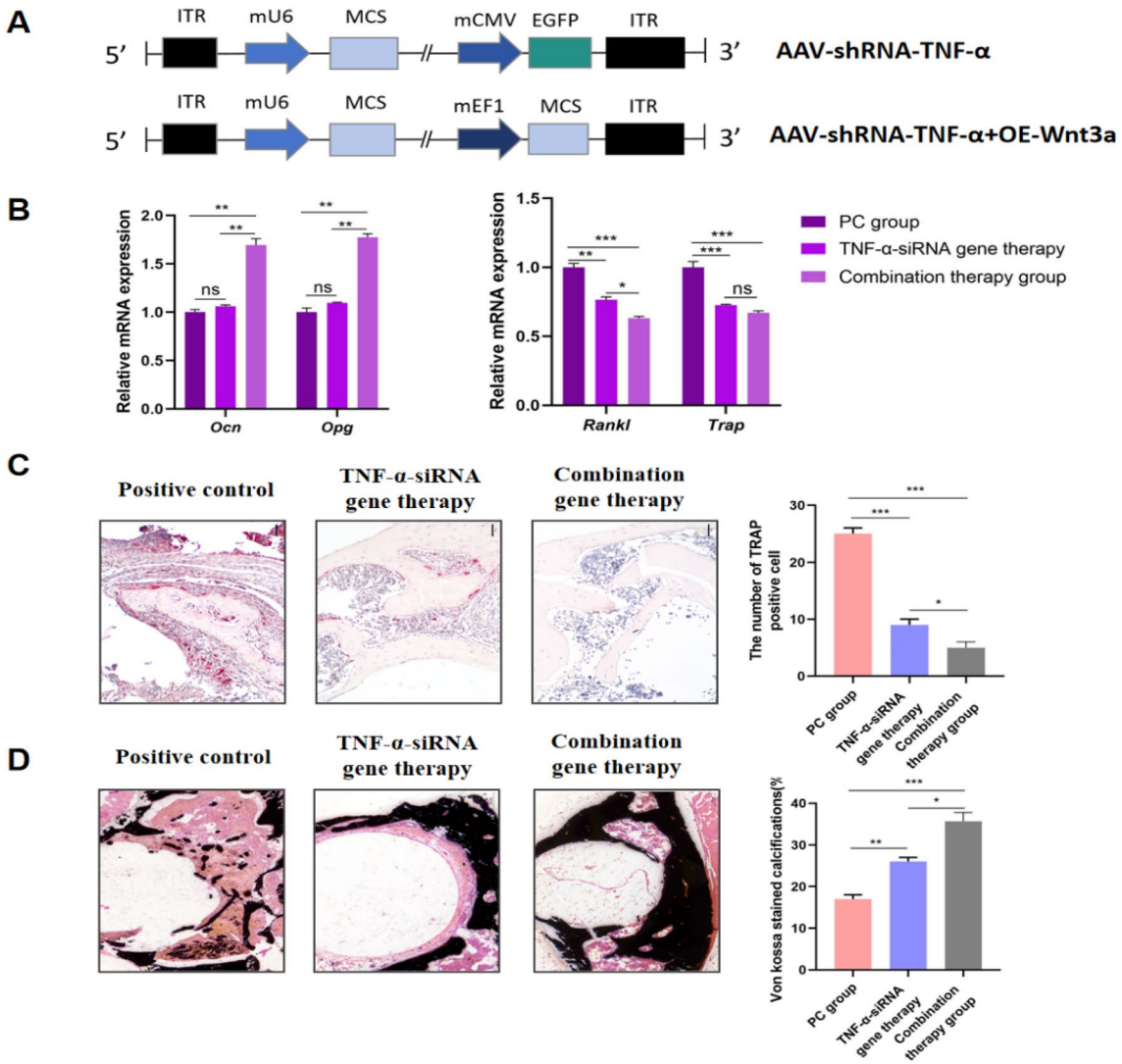

Fig. 4 Comparison of the effectiveness of monotherapy and combination therapy. **A** Schematic diagram of recombinant adeno-associated vectors. **B** mRNA expression levels of osteogenic genes Ocn, Opg (left) and osteoclast genes Rankl, Trap (right) after AAV-mediated gene therapy. **C** TRAP immunohistochemical staining and quantification. **D** Von kossa staining and calcification quantification results of knee joint. ***P < 0.001, **P < 0.01, and *P < 0.05

(Ocn and Opg) were significantly higher in the combination therapy group compared to the monotherapy group, while osteoclast-related genes (Rankl and Trap) were further downregulated (Fig. 4B), suggesting that combination therapy had a superior therapeutic effect.

To further evaluate the efficacy of combination therapy, we performed TRAP staining to assess osteoclast numbers. The proportion of TRAP-positive osteoclasts decreased after gene therapy, particularly in the combination therapy group (Fig. 4C). Von Kossa staining revealed an increased calcified area in the combination therapy group compared to the monotherapy group (Fig. 4D). In addition, urinary deoxypyridinoline (DPD) levels, a marker of bone metabolism, were higher in the PC group, indicating more severe bone destruction (Supplementary Fig. 4C). After combination therapy, DPD levels were significantly reduced, demonstrating effective mitigation of bone destruction. Overall, the combined targeting of *Wnt3a* and *TNF-α* showed a more pronounced therapeutic effect than monotherapy.

Discussion

This study sheds light on the complex mechanisms by which titanium particles contribute to osteolysis and highlights the potential of gene therapy in addressing prosthesis loosening. The findings underscore the

importance of targeting both the inflammatory response and the Wnt signaling pathway in developing novel therapeutic strategies.

In vitro and in vivo experiments demonstrated that titanium particles exert a dual influence on osteolysis. On one hand, they stimulate the differentiation of RAW264.7 cells into osteoclasts, as evidenced by increased TRAP-positive cells and upregulated osteoclastogenesis markers (Figs. 1A, 4B). This aligns with previous research showing that wear particles activate macrophages, leading to enhanced bone resorption [25, 26]. On the other hand, titanium particles inhibit the differentiation of BMSCs into osteoblasts, as indicated by reduced ALP activity and downregulated osteoblastogenic markers (Figs. 2B, C, 4B). This inhibition reflects the dysregulation of bone remodeling observed in periprosthetic osteolysis, where bone resorption is elevated and bone formation is suppressed (Fig. 4C, D).

The pro-inflammatory effects of titanium particles are further emphasized by the elevated levels of cytokines, such as *TNF-α*, IL-6, and IL-1β. This inflammatory response is a central mediator of osteolysis, stimulating osteoclast activity while suppressing osteoblast function, resulting in net bone loss. RNA-seq analysis revealed the involvement of TNF signaling and NF-κB pathways, which are integral to the inflammatory response and osteoclast differentiation (Fig. 2E). The Wnt signaling pathway, particularly the Wnt/β-catenin pathway, plays a crucial role in osteoblast differentiation and bone formation [27, 28]. Our results indicate that titanium particles downregulate Wnt pathway-related factors, including *Wnt3a* and β-catenin, suggesting a mechanism by which titanium particles inhibit osteoblastogenesis. This is consistent with the decreased expression of osteoblast markers and impaired bone formation observed in our models.

The therapeutic potential of gene therapy targeting *TNF-α* and *Wnt3a* was evaluated in a mouse model of knee prosthesis loosening. Combined gene therapy outperformed monotherapy in reducing inflammatory cell infiltration, promoting bone formation, and mitigating bone destruction. This was demonstrated by reduced TRAP-positive osteoclasts, increased calcified areas in von Kossa staining, and decreased urinary DPD levels in the combination therapy group. These findings suggest that simultaneously targeting inflammatory factors and the Wnt signaling pathway could provide a synergistic approach to alleviating prosthesis loosening.

The success of gene therapy in this study highlights its potential as a novel treatment strategy for aseptic prosthesis loosening. By addressing both the inflammatory response and promoting osteoblast differentiation, gene therapy offers a comprehensive approach to managing this complex condition. The use of AAV-mediated gene delivery demonstrated efficacy, paving the way for clinical translation. To fully understand the long-term effects and clinical applicability of these findings, future studies should focus on refining gene therapy techniques, exploring additional targets within the inflammatory and osteogenic pathways [29–31], and conducting clinical trials to assess the safety and efficacy of these treatments in patients with prosthesis loosening. In addition, research into biomaterials and implant designs that minimize wear debris generation and its effects on bone tissue is warranted. By continuing to explore these avenues, we can improve the longevity and success of joint replacement surgeries and enhance the quality of life for patients suffering from osteoarthritis and other joint disorders.

Conclusion

This study reveals the dual role of titanium particles in osteolysis and underscores the potential of gene therapy in improving knee prosthesis loosening. By targeting both inflammation and osteogenic pathways, this approach offers a promising strategy for managing aseptic prosthesis loosening in the future.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40001-025-02452-3.

Supplementary material 1

Supplementary material 2

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Not applicable.

Author contributions
KNS, QHJ and PC conceptualized the study. PC, HHG, LW, SZ collected the data. KNS, QHJ and PC drafted the manuscript. All authors participated in designing the study, interpreting and analyzing the data, and reviewing the manuscript and approved the final version of the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. All procedures concerning the animals use and care were approved by the Animal Research-Animal Care Committee of the General Hospital of Ningxia Medical University, and was conducted in accordance with the guidelines issued by the committee. The approval document number for this animal experiment is 2020–913.

Consent for publication
All authors have agreed to the publication of this manuscript.

Competing interests
The authors declare no competing interests.

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