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Yeast microcapsule-mediated oral delivery of IL-1β shRNA for post-traumatic osteoarthritis therapy

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Post-traumatic osteoarthritis is a prevalent debilitating joint disease. However, there is no FDA-approved disease-modifying osteoarthritis drug currently. Gene therapy can improve disease progression but lacks an effective delivery system. Here, we constructed an oral drug delivery system by non-virus-mediated interleukin-1β (*IL-1β*) short hairpin RNA (shRNA) and non-pathogenic yeast to evaluate its effect on osteoarthritis therapy. After recombinant *IL-1β* shRNA/yeast therapy, yeast microcapsule-mediated oral delivery of *IL-1β* shRNA greatly reduced the IL-1β expression in intestine macrophage, bone marrow macrophage, and articular cartilage, systematically regulate the inflammatory response. The degeneration of articular cartilage was significantly inhibited in the medial femoral condyle and medial tibial plateau of the knee joint. And the expression of osteoarthritis markers Col X and MMP13 was reduced in the knee joint. Thus, yeast microcapsule-mediated oral delivery of *IL-1β* shRNA may serve as a novel gene therapy strategy for treating joint degeneration through immunomodulation of the mononuclear phagocyte system from the intestine to subchondral bone marrow and ultimately preserving the articular cartilage joint.

INTRODUCTION

Post-traumatic osteoarthritis (PTOA) is a prevalent degenerative joint disease affecting 5.6 million people in the US every year.[1] PTOA is characterized by an acute injury-induced joint inflammation followed by a gradual degradation of articular cartilage joint.[2,3] Anterior cruciate ligament and meniscus surgery may restore joint function but does not affect PTOA pathogenesis.[4,5] There is no FDA-approved disease-modifying osteoarthritis drug for prevention and treatment of PTOA.[6] A barrier of developing a PTOA drug is the lack of an effective drug-delivery system.

Interleukin-1β (*IL-1β*) is one of the key genes involved in the pathogenesis of PTOA.[7] It can promote the production of matrix metalloproteinases (MMPs) and enhance collagen and proteoglycan breakdown in cartilage.[8,9] Therefore, inhibiting *IL-1β* has been proposed as a promising therapy for the treatment of PTOA. IL-1 receptor antagonist *(IL-1RA)* can be used to regulate *IL-1β* expression, but its half-life is very short.[10] Furthermore, administration of *IL-1RA* requires intra-articular injection.[2] Such injection with short intervals is not practical for managing a long-term degenerative disease such as PTOA. Gene therapy using *IL-1RA* has shown its promise, but it requires viral vectors for delivery, and a clinical trial to assess its safety is underway.[11]

Oral drug administration is a preferred route due to its ease of use, low cost, and high patient compliance. Compared to parenteral administration, oral route typically causes neither tissue damage nor pain and requires less patient supervision and cost of care.[12] Despite the advantages of oral administration, oral uptake of many bio-therapeutics is limited by various physiological barriers such as the harsh gastrointestinal environment and enzymatic degradation and tolerance, which impede the clinical application of oral delivery systems. Although many studies have demonstrated that bacteria and virus can be used for drug delivery including short hairpin RNA (shRNA) gene therapy,[13–15] their use has been limited due to safety concerns.[16] Moreover, viruses have limited packaging capacity,[17] and bacteria have a low survival rate in the stomach and small intestine.[16]

Saccharomyces cerevisiae has been shown to be resistant to digestion in the stomach and small intestine[18] and is widely used in wine making.[19] One of the reasons for interest in yeast microcapsule as a vaccine vehicle is its low toxicity with a maximum tolerated dose in several clinical trials.[20,21] The other advantage of yeast microcapsule is its property of targeting antigen presenting cells (APCs) including macrophages and dendritic cells in the gastrointestinal tract. Beta-glucans, carbohydrate polymers on the yeast cell wall,[18,22] could be recognized by glucan receptors on the surface of the macrophages.[23] After yeast microcapsule is engulfed by macrophages in the gut-associated lymphatic tissue (GALT), the GALT macrophages may traffic away from the gut, infiltrate other mononuclear phagocyte system tissues including bone and bone marrow, and modulate the production of pro-inflammatory cytokines at these distant tissue sites.[24,25] Thus,

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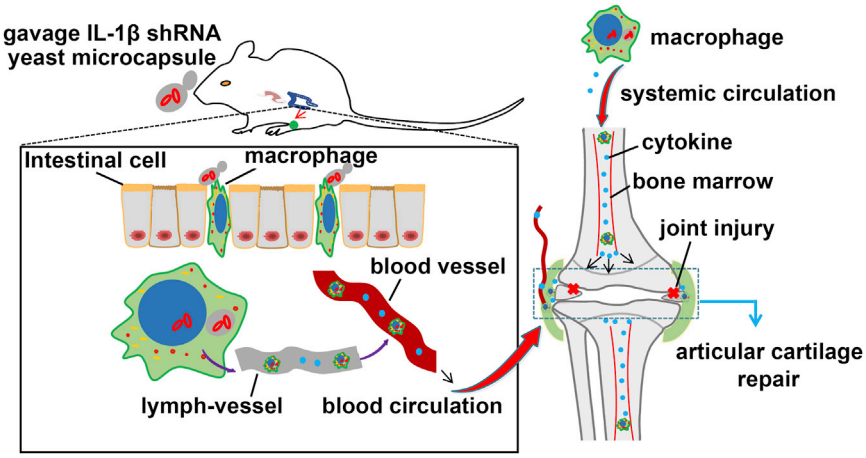

Figure 1. Schematic diagram of oral gene therapy for PTOA with recombinant *IL-1β* shRNA yeast microcapsule

After oral administration of recombinant *IL-1β* shRNA yeast microcapsule, *IL-1β* shRNA is delivered to the small intestine by passing through the intestinal epithelium.[24] Yeast microcapsule will be recognized and engulfed by macrophages.[26] Macrophages and cytokines that are induced by yeast microcapsule enter the humoral circulation and transfer to other mononuclear phagocytic systems such as bone marrow and synovium.[25] By modulating the joint immune environment, macrophages and cytokines inhibit the inflammatory response and catabolic pathway and stimulate the anabolic pathway to control PTOA pathogenesis.

these characteristics make yeast microcapsule a preferred delivery vehicle for disease treatment by oral administration.

In this study, we hypothesized that yeast microcapsule-mediated oral delivery of *IL-1β* shRNA can be used for PTOA therapy (Figure 1). The aim of the present study was to develop a safe and efficient micro-drug-delivery system for chronic diseases treatment not limited to PTOA therapy.

RESULTS

To develop an oral gene therapy for PTOA, we formulated the following strategy: (1) screen and identify shRNAs that could inhibit *IL-1β* expression in mammalian cells; (2) generate recombinant yeast strains that harbor *IL-1β* shRNA plasmids; (3) use *IL-1β* shRNA recombinant yeast to target the lipopolysaccharide (LPS) induced macrophages to detect the mechanism of the study *in vitro*; (4) orally administer recombinant yeast microcapsule to the PTOA model mice; (5) detect *IL-1β*- and osteoarthritis (OA)-related gene expression in bone marrow macrophages and articular cartilage; 6) detect IL-1β and downstream cytokine expression in systemic immunomodulation in serum; and (7) evaluate articular cartilage repair by histological staining.

Functional testing of *IL-1β* shRNA *in vitro*

To construct *IL-1β* shRNA vector, we selected three targeted sequences in the mouse *IL-1β* gene based on bioinformatics analysis: shRNA533 targeting exon 5 and shRNA1037 and shRNA1233 targeting two sites in exon 7, respectively (Figure 2A). The shRNA expression vector (pIN27-hU6-shRNA-miR30) contains the hU6 promoter to drive shRNA expression and two miR-30 flanking sequences to enhance its expression, as we previously described.[18] To determine whether shRNA expression vectors functioned in mammalian cells, the shRNA vector was transfected into 293T cells with the reporter vector pRP-CMV-IL-1β-GFP that contained the IL-1β-GFP fusion cassette driven by the CMV (Cytomegalovirus) promoter (Figure 2B). The intensity of green fluorescence reflects the degree of IL-1β expression. If *IL-1β* shRNA binds to the IL-1β targeted sequence, the expression of the IL-1β-GFP fusion gene would be knocked down, and the GFP green fluorescence would be reduced. Compared with the control group (pIN27-hU6-miR30 with negative control shRNA expression cassette), the intensity of green fluorescence was weakened in shRNA533, shRNA1037, and shRNA1233 groups (Figures 2C and 2D). Thus, the *IL-1β* shRNAs have the ability to knock down IL-1β expression in mammalian cells.

Generation of *IL-1β* shRNA recombinant yeast

Recombinant yeast strains that contain these three *IL-1β* shRNA and the negative control vector were generated by transformation of yeast with plasmids pIN27-hU6-shRNA-miR30 or pIN27-hU6-miR30 (control), respectively. Recombinant shRNA yeast was screened with selective medium (Figure S1). Due to the specific expression of uracil in shRNA vector (Figure 2B), only recombinant yeast containing shRNA vector can survive in uracil-deficient medium. Plasmid pIN27-hU6-shRNA-miR30 or pIN27-hU6-miR30 (1 μg) was transformed into Scy27 yeast by the LiAc (Lithium acetate) method. The shRNA transformation rate of Scy27 was about $(9.5 \pm 0.64) \times 10^2$ cfu/μg (Figure S2). PCR detection of single yeast clone with shDec-F and shDec-R primers (Table 2) indicated that the recombinant yeast contained the shRNA at the predicted length (Figure S3), which was subsequently confirmed by sequencing (data not shown). Thus, four recombinant yeast strains were generated.

Recombinant *IL-1β* shRNA yeast downregulate *IL-1β* expression in macrophages *in vitro*

In order to prove recombinant yeast can be recognized and phagocytized by macrophages, we used green aminofluorescein-labeled yeast microcapsule to target the LPS-induced RAW264 macrophages *in vitro*. The results showed that green fluorescence-labeled yeast microcapsule (Figure 3A) was engulfed by macrophages (Figure 3B), and the phagocytosis process was recorded by dynamic video (Video S1, Macrophage uptake yeast microcapsules).

To detect the function of shRNA recombinant yeast on macrophages, we used *IL-1β* shRNA recombinant yeast to target the LPS-induced RAW264 macrophages. Gene expression of *IL-1β*, *Nr1D2*, tumor

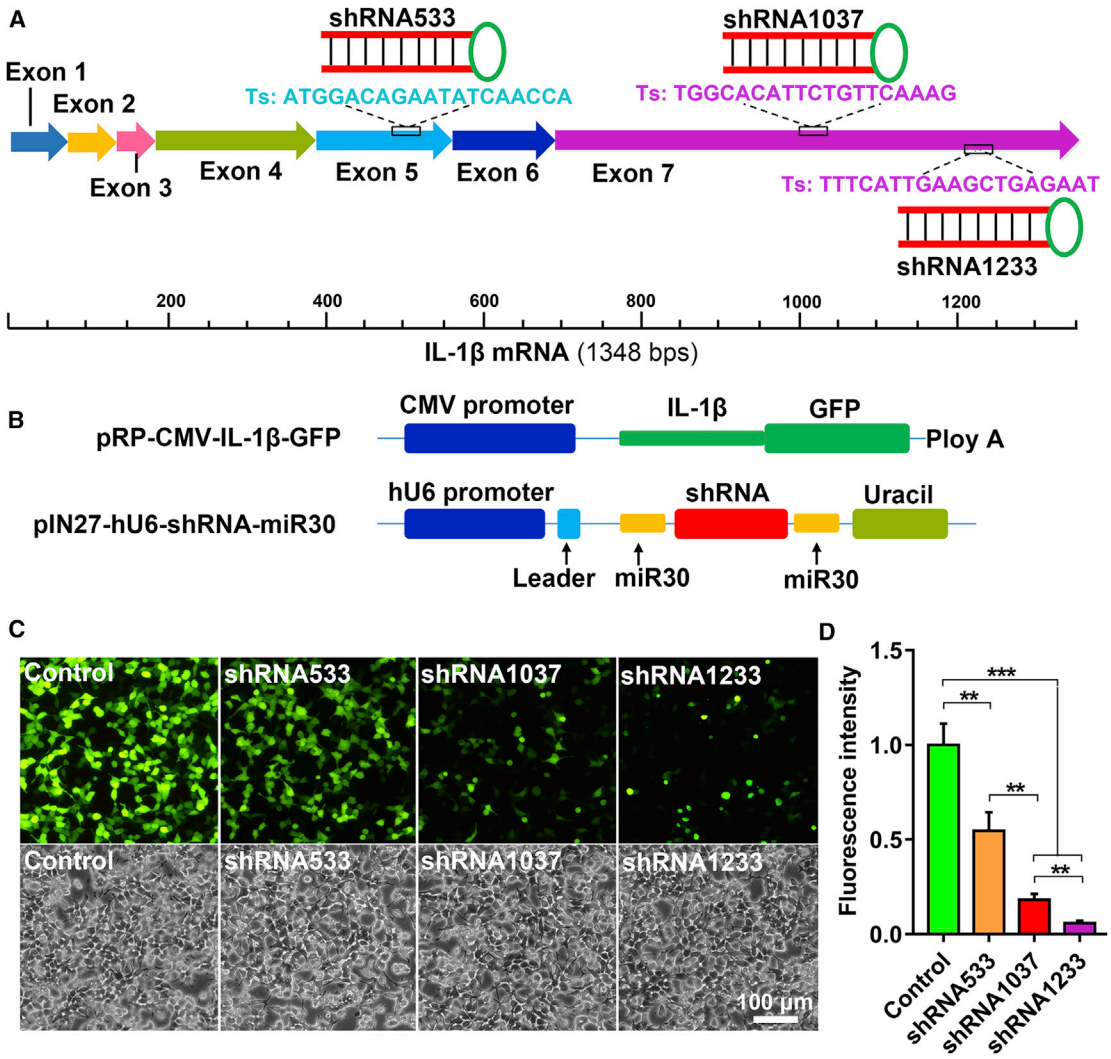

Figure 2. The structure of shRNA expression vector and functional detection of *IL-1β* shRNA in 293T cells *in vitro*

(A) shRNA target sites and target sequence (Ts) on the IL-1β mRNA. (B) The structure of pRP-CMV-IL-1β-GFP (pRP) and pIN27-hU6-shRNA-miR30 plasmid. (C) Functional detection of IL-1β shRNA in 293T cells. The shRNA expression vectors were co-transfected into 293T cells with the reporter vector pRP-CMV-IL-1β-GFP that contained a GFP expression cassette driven by the CMV promoter. After 24 h treatment, green fluorescence was detected by the fluorescent microscope. (D) Fluorescence intensity in each group was quantified. Data were expressed as mean ± SD (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$.

necrosis factor alpha (*TNF-α*), and arginase-1 were detected by qRT-PCR. Compared with the control group (yeast containing pIN27-hU6-miR30), the expression of *IL-1β* and *TNF-α* in the shRNA groups was downregulated, while *Nr1D2* and arginase-1 were upregulated (Figure 3C). These results showed that *IL-1β* shRNA recombinant yeast could alleviate inflammation by reducing the expression of inflammatory factors. These results provide the most fundamental guarantee for *IL-1β* shRNA recombinant yeast to be used in PTOA therapy *in vivo*.

Yeast is enriched in the joint injury site after oral administration

In order to detect whether yeast can transfer to the joint injury site after oral administration, we gave the joint injury model mice oral administration of near-infrared fluorescence-labeled rYeast (Figure 4A) or yeast (control). Compared with the control group (non-fluorescent-labeled yeast), the fluorescence signal could be detected (Figure 4A) in the Odyssey imaging system at different dilution concentrations (1, 1/2, 1/4, and 1/8). After continuous administration 3 times (30 mg/kg rYeast or yeast every day), the knee joints of mice were collected for fluorescence imaging detection using the Odyssey CLX imaging system. Compared with the control group (oral administration of non-fluorescent-labeled yeast), a large number of fluorescence signals were detected in the rYeast-treated mouse joints (Figures 4B and 4C). Evidence suggests that yeast could be engulfed by GALT macrophages via an oral route. And macrophages in the GALT may traffic away

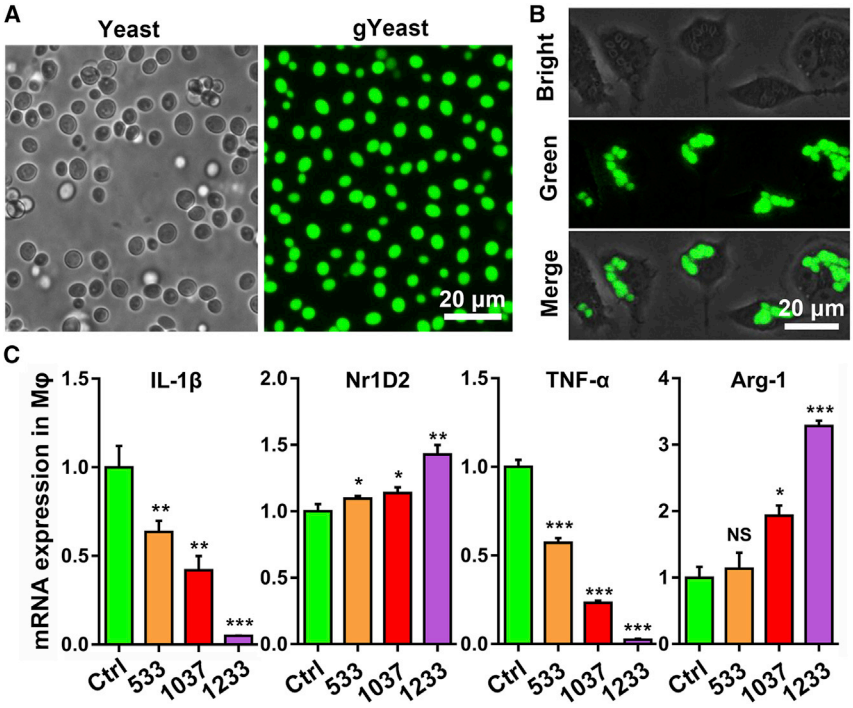

Figure 3. Yeast be engulfed by macrophages and can regulate the target gene expression.

(A) Aminofluorescein-labeled yeast microcapsule. (B) Phagocytosis of yeast microcapsules by macrophages. (C) Targeting of the LPS-induced macrophages with IL-1β shRNA recombinant yeast *in vitro*. The gene expressions of *IL-1β*, *Nr1D2*, *TNF-α*, and arginase-1 were detected by qPCR. Data was expressed as mean ± SD. NS, no significance; *p < 0.05, **p < 0.01, ***p < 0.001 versus control group (n = 3).

from the gut and infiltrate other mononuclear phagocyte systems and inflammatory tissues.[24,25,27,28] This indicated that rYeast was engulfed by intestinal macrophages and indeed transferred from the gut to the knee joint.

Recombinant *IL-1β* shRNA yeast downregulate intestinal inflammatory response

PTOA mouse model was generated by surgical destabilization of the medial meniscus (DMM), as described in the Materials and methods. One month after surgery, the DMM mice were fed with 30 mg/kg (per mouse) recombinant *IL-1β* shRNA yeast every two days for 40 days. Macrophages from small intestine were isolated through CD11b affinity purification. As one of the main organs of drug absorption, we measured the IL-1β expression of intestinal macrophages. Compared with the control group (ctrl, shRNA control), the expression of IL-1β was inhibited in the experimental mice (shRNA groups) at different degrees (Figure S4), and the intestinal immunofluorescence showed that the expression of IL-1β and TNF-α in the shRNA1233 group was significantly inhibited (Figure S4A). These results showed that yeast microcapsule-mediated targeted delivery of *IL-1β* shRNA successfully downregulated the intestinal inflammatory response in PTOA mice.

Recombinant yeast regulate systemic immune response

Cytokines can be used for systemic immune regulation. To determine whether cytokine secretion was affected by recombinant IL-1β shRNA yeast, here we quantify the expression of IL-1β, TNF-α, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-21 in serum. Serum IL-1β levels were significantly inhibited in *IL-1β* shRNA groups (Figure 5). shRNA1233 achieved the largest inhibition, while shRNA533 achieved the smallest inhibition. All three *IL-1β* shRNAs inhibited the expression of cytokines TNF-α, GM-CSF, and IL-12. While shRNA1233 did not alter IL-21 serum levels significantly, shRNA533 and shRNA1037 increased IL-21 levels significantly (Figure 5). Although IL-10 was usually considered as an anti-inflammatory cytokine, the expression of IL-10 in serum of IL-1β shRNA-treated mice was decreased. This may be due to the fact that the inflammatory factors and anti-inflammatory factors have reached a dynamic balance in the later stage of shRNA treatment. This suggested that recombinant yeast can regulate inflammatory response systematically.

Downregulation of OA-related gene expression in bone marrow macrophages and articular joints

As systemic immune regulators, inflammatory cytokines were inhibited by recombinant *IL-1β* shRNA yeast. Since recombinant *IL-1β* shRNA yeast could downregulate the local (small intestine) and systemic (cytokines in serum) inflammatory response in PTOA mice, it was unknown whether it could regulate the gene expression in articular cartilage and subchondral bone marrow. Here, we not only quantified *IL-1β* mRNA levels in small intestine macrophages, but also in bone marrow macrophages and knee joint tissues (Figure 6). The results showed that *IL-1β* was downregulated in small intestine macrophages, bone marrow macrophages, and knee joints. Thus, oral administration of recombinant *IL-1β* shRNA yeast could effectively inhibit *IL-1β* mRNA levels in intestine macrophages. They can also effectively inhibit the expression of *IL-1β* in bone marrow (BM) macrophages and knee joints.

PTOA is associated with cell inflammation and senescence.[7] *Nr1D2* (REV-ERBβ) is a circadian clock gene involved in transcription repression of inflammation.[29] The expression levels of *Nr1D2*, a transcription suppressor of inflammation, were stimulated significantly by oral administration of shRNA1233 and shRNA1037 in intestine macrophages, bone marrow macrophages, and knee joints (Figure 6). To verify whether the anti-inflammatory activity of recombinant *IL-1β* shRNA yeast was related to macrophage polarization, we quantified M1 marker *TNF-α* and M2 marker arginase-1 (*Arg-1*) in intestine and bone marrow macrophages. Both of *TNF-α* and *Arg-1* were

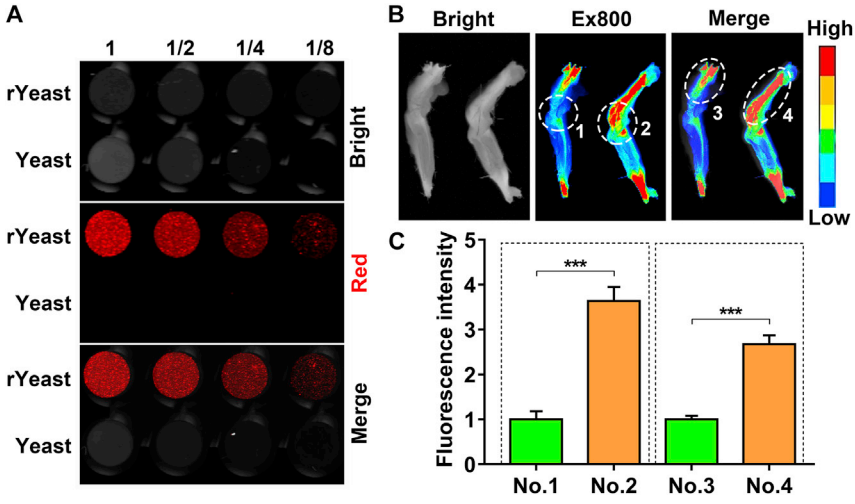

Figure 4. Yeast phagocytized by macrophages was enriched in the joint injury site

(A) Odyssey CLX imaging showed that yeast was successfully labeled with near-infrared Dir fluorescent dye (rYeast refers to near-infrared fluorescence-labeled yeast). (B) Fluorescence intensity in the knee joint (1 and 2; left, yeast; right, rYeast) and femur bone marrow cavity (3 and 4; left, yeast; right, rYeast) was measured by Odyssey CLX. (C) Fluorescence intensity in each group was quantified. Data were expressed as mean ± SD. ***$p < 0.001$ ($n = 3$).

downregulated in macrophages (Figures 6A and 6B). Oral delivery of *IL-1β* shRNAs significantly elevated anabolic cartilage matrix marker col2a1 (*Col II*) level, while suppressing hypertrophic marker col10a1 (*Col X*) levels in cartilage joint (Figure 6C).

Recombinant *IL-1β* shRNA yeast has obvious effects on articular cartilage repair

Hematoxylin and eosin (H&E) and safranin O/fast green staining were used to evaluate the therapeutic effect of recombinant *IL-1β* shRNA yeast on PTOA from the perspective of pathology. DMM surgery caused cartilage loss, and depletion of proteoglycan was effectively inhibited by recombinant yeast (Figures 7A and 7B). The degree of osteoarthritic damage was quantified using Osteoarthritis Research Society International (OARSI) criteria. Osteoarthritis damage scores of the medial tibial plateau (MTP) and medial femoral condyle (MFC) showed that three shRNAs achieved significantly lower grades than those of the control group (Figure 7C). The *IL-1β* shRNA1233 group achieved the lowest (best) OARSI score, with almost complete protection of the MTP articular cartilage. OA marker MMP13 was highly expressed by articular chondrocytes in the control treatment group, but its expression was greatly inhibited in IL-1β shRNA treatment groups (Figures 7D and 7E), and the knee joint immunofluorescence showed that the expression of IL-1β (green) in the shRNA1233 group was significantly inhibited (Figure 7F). Therefore, oral administration of recombinant *IL-1β* shRNA yeast can alleviate degradation of articular cartilage and has therapeutic effect on joint injury.

DISCUSSION

PTOA is characterized by an acute injury-induced joint inflammation followed by a gradual degradation of articular cartilage joints.[2,3] Currently, there is no FDA-approved disease-modifying osteoarthritis drug, and the final treatment of OA is joint replacement.[6] Compared to parenteral administration, oral route typically causes neither tissue damage nor pain and requires less patient supervision and cost of care.[12] Although some oral drugs, such as acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), and glucosamine/chondroitin sulfate,[30] could lessen OA joint pain, they cannot affect OA disease progression.

Here, we developed a novel strategy for PTOA therapy via yeast microcapsule-mediated orally targeted delivery of *IL-1β* shRNA, which could regulate systemic inflammatory response to achieve local tissue inflammation treatment. There are some reasons for choosing yeast as an oral-delivery vehicle. First, as brewer's/baker's yeast, *S. cerevisiae* is safe and widely used in food, wine, and beer making. Second, we previously showed that yeast can be used for shRNA delivery and resistant to digestion in the stomach and small intestine.[18] In addition, yeast delivery of nucleic acid into cells avoided undesired gene integration and tumorigenesis.[31,32] The other advantage of yeast is its property of targeting APCs such as macrophages in the gastrointestinal tract.[33] Beta-glucans, carbohydrate polymers on the yeast cell wall,[18,22] could be recognized by glucan receptors on the surface of macrophages, which is consistent with our results (Figure 3B; Video S1). This enables yeast to achieve immune regulation at the same time of gene delivery.

IL-1β is one of the key genes involved in the pathogenesis of PTOA.[7] It can promote the production of MMPs and enhance collagen and proteoglycan breakdown in cartilage.[8,9] Previous study showed that viral-mediated *IL-1R* antagonist cDNA achieved disease-modifying effects *via* intra-articular administration.[34,35] However, gene therapy with non-viral approaches was difficult due to the low efficiency of delivery. In this study, we developed a strategy to inhibit IL-1β expression by using shRNA interference. This approach has been shown to knock down target gene expression with high specificity.[36] We designed three *IL-1β* shRNA to target *IL-1β* mRNA, respectively (Figure 2A). The miR30-based shRNA expression vector was driven by the U6 promoter along with a small nuclear RNA (snRNA) leader sequence (Figure 2B), which showed high transfection efficiency without virus mediation.[37] Our data indicated that the *IL-1β* shRNA constructed via this method were effective in knocking down IL-1β expression both in 293T cells (Figure 2C) and macrophages (Figure 3C).

In this study, mice were fed with recombinant *IL-1β* shRNA yeast for 40 days, starting from one month after the DMM surgery. This was to ensure the anti-IL-1β therapeutics' targeting the tissue degeneration

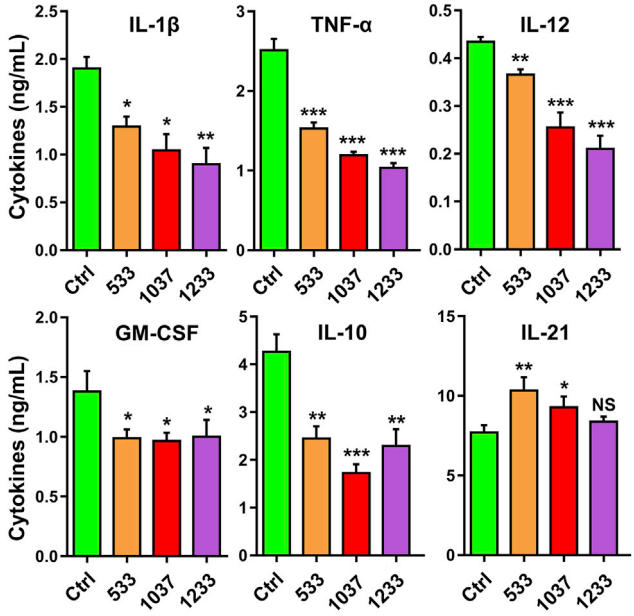

Figure 5. Cytokine expression in serum

With oral administration 30 mg/kg recombinant yeast every other day for 40 days, serum samples were harvested from the tail veins. The cytokines of IL-1β, TNF-α, IL-12, GM-CSF, IL-10, and IL-21 in the serum were analyzed. Data were expressed as mean ± SD. NS, no significance; *p < 0.05, **p < 0.01, ***p < 0.001 versus ctrl group (n = 6).

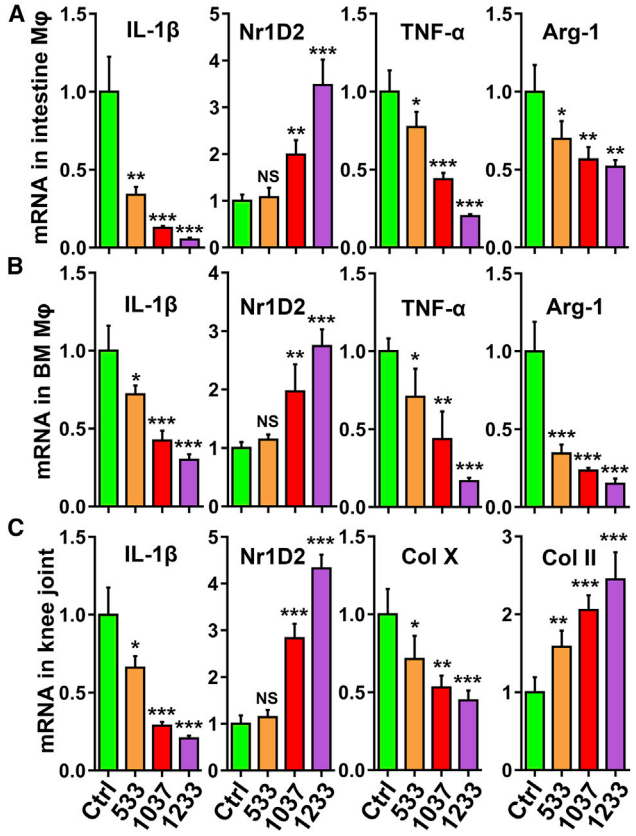

Figure 6. Detection of *IL-1β* and PTOA-related functional gene expression *in vivo*

(A–C) With oral administration 30 mg/kg recombinant yeast every other day for 40 days, RNA was extracted from intestine macrophages (A), bone marrow macrophages (B), and knee joint (C) to detect PTOA-related functional gene expression. Data was expressed as mean ± SD. NS, no significance; *p < 0.05, **p < 0.01, ***p < 0.001 versus ctrl group (n = 6).

process but not the initial injury response. The expression of IL-1β in small intestinal macrophage was downregulated with the treatment of recombinant *IL-1β* shRNA yeast (Figure S4). And the intestinal immunofluorescence showed that the expression of *IL-1β* and *TNF-α* in the shRNA1233 group was significantly inhibited (Figure S4A). These results showed that yeast microcapsule-mediated targeted delivery of *IL-1β* shRNA successfully downregulated the intestinal inflammatory response in PTOA mice.

Although OA was considered as a non-inflammatory local disease, more and more evidence indicated that OA can be a systemic disease involving multiple organs and inflammatory status, which are associated with aging, injury, and diet.[38,39] For example, OA can be induced by a high-fat diet, which exerts a systemic inflammatory effect.[40] Such an OA-causing effect involves IL-1β and other major cytokines.[41] Our data proved that the attenuation of OA pathogenesis by oral-targeted delivery of *IL-1β* shRNA yeast microcapsules also involves systemic immune modulation of cytokines. Serum levels of IL-1β, TNF-α, IL-10, IL-12, and GM-CSF were downregulated and IL-21 was upregulated (Figure 5). These cytokines play significant roles in many diseases, signaling pathways,[42] and immune responses.[43] Inflammatory cytokines TNF-α, GM-CSF, interferon-γ (IFN-γ), and IL-21 have been confirmed to be associated with OA.[39,44] Although IL-10 is usually considered as an anti-inflammatory cytokine, its levels are increased in OA joints, suggesting IL-10 and IL-1β may be co-regulated.[45] Drugs targeting macrophages and macrophage-associated inflammatory pathways can improve the development of PTOA. This conclusion was supported by the recent finding that activated macrophages are present in a substantial proportion of human OA knee joints and that the presence of activated macrophages in the joint was associated with OA severity and joint symptoms.[46] This suggests that recombinant *IL-1β* shRNA yeast can systematically regulate the inflammatory response in PTOA mice.

As systemic immune regulators, inflammatory cytokines were inhibited by recombinant *IL-1β* shRNA yeast. Recombinant *IL-1β* shRNA yeast can downregulate the local (small intestine) and systemic (cytokines in serum) inflammatory response in PTOA mice. We speculate that the downregulation of the systemic inflammatory response has a certain effect on the repair of pathological tissue. Our results showed that downregulation of IL-1β expression results in a series of changes, including downregulation of inflammatory cytokines, hypertrophic markers Col X and MMP13, and

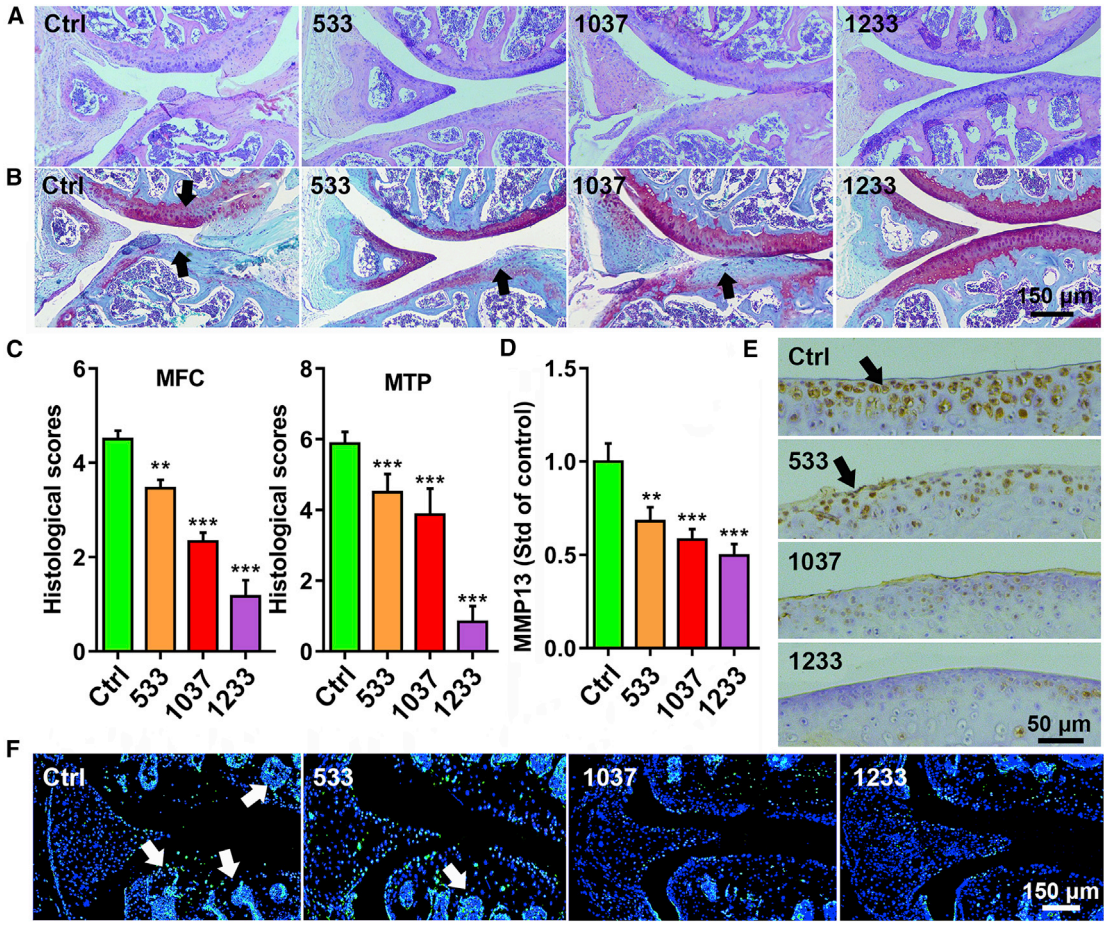

Figure 7. Recombinant *IL-1β* shRNA yeast has obvious effect on articular cartilage repair

(A, B, E, and F) Histological images of osteoarthritic cartilage were stained by H&E (A), safranin O/fast green (B), immunohistochemistry (E), and immunofluorescence (F). (C) Osteoarthritic damage scores of the MTP and MFC were assessed using OARSI criteria. (D) MMP13 mRNA expression in articular cartilage. (E) Immunohistochemistry of OA marker MMP13. (F) The expression of IL-1β (green) in knee joint was detected by immunofluorescence. DAPI nuclear staining (blue) was also conducted. Inhibition of IL-1β expression with *IL-1β* shRNA recombinant yeast alleviated proteoglycan loss, structure damage, MMP13, and IL-1β expression as indicated by arrow (n = 6). Data were expressed as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

upregulation of anabolic marker Col II. The inflammatory response in articular cartilage was inhibited, and subsequent histological staining confirmed our speculation (Figure 7). Therefore, oral administration of recombinant *IL-1β* shRNA yeast can alleviate degradation of articular cartilage and has therapeutic effect on joint injury.

In conclusion, we created a novel and efficient shRNA delivery system via orally administered recombinant yeast for PTOA therapy and immunomodulation. We demonstrate the feasibility of PTOA treatment via yeast microcapsule-mediated oral gene therapy. Such approach may have strong potentials for treatment of inflammatory and degenerative diseases not limited to PTOA, as demonstrated here. Thus, yeast microcapsule-mediated oral delivery of shRNA may serve as a new gene therapy strategy for joint-degeneration treatment.

MATERIALS AND METHODS

Construction of *IL-1β* shRNA expression vectors

Three different *IL-1β* shRNA sequences were designed and their target sites were shown in Table 1. The yeast cloning vector pIN27-hU6- *IL-1β*-shRNA was constructed as we previously described.[18]

Functional detection of *IL-1β* shRNA *in vitro*

293T cells were employed to investigate shRNA function. In brief, the shRNA expression vectors were co-transfected into 293T cells with the reporter vector pRP-CMV-*IL-1β*-GFP that contained a GFP expression cassette driven by the CMV promoter. Cells were incubated at 37°C under 5% CO_2 for 24 h. Green fluorescence was detected by the fluorescent microscope Leica Dmil Led (Leica, Wetzlar, Germany).

Table 1. The sequences of IL-1β shRNA and its target sites

shRNA name	Sequence (5′ to 3′)	Target site (5′ to 3′)
IL-1β shRNA533	TGCTGTTGACAGTGAGCGCG**GACAGAATATCAACCAACA**ATA*GTGAAGCCACAGATG* TAT**TGTTGGTTGATATTCTGTC**CCATGCCTACTGCCTCGGA	ATGGACAGAATATCAACCA
IL-1β shRNA1037	TGCTGTTGACAGTGAGCGAG**CACATTCTGTTCAAAGAGA**GTA*GTGAAGCCACAGATG* TAC**TCTCTTTGAACAGAATGTG**CCTGCCTACTGCCTCGGA	TGGCACATTCTGTTCAAAG
IL-1β shRNA1233	TGCTGTTGACAGTGAGCGCT**CATTGAAGCTGAGAATAAA**TTA*GTGAAGCCACAGATG* TAA**TTTATTCTCAGCTTCAATG**AATGCCTACTGCCTCGGA	TTTCATTGAAGCTGAGAAT

Bold sequence refers to the functional sequence of shRNA, and italic sequence refers to the loop structure of shRNA.

Construction of *IL-1β* shRNA recombinant yeast

The yeast strain Scy27 (*MATα, his3-Δ1 trp1-289 rad1-Δ ura3-52*) was used in this study. Plasmids pIN27-hU6-shRNA-miR30 and pIN27-hU6-miR30 (control vector without shRNA expression cassette) were respectively transformed into Scy27 by the LiAc method. Single yeast clones were picked and cultured in the selective liquid medium until reaching a density of optical density 600 (OD_{600}) = 1.0. Cells were harvested and re-suspended with PBS and stored at −20°C until oral administration.

Yeast microcapsule is engulfed by macrophages

In order to prove that yeast microcapsule can be recognized and engulfed by macrophages, we labeled yeast with 5-(4,6-dichlorotriazinyl) aminofluorescein (Thermo; 1 mg/mL in DMSO) to construct green-labeled yeast microcapsule as described by Aouadi et al.[27] All the other chemical reagents used here were purchased from Sigma. Then the green-labeled yeast microcapsule was harvested and stored at −20°C before use. RAW264 macrophages were cultured for 24 h in DMEM (ATCC: 30-2002) containing 10% fetal bovine serum (FBS) supplemented with 100 ng/mL LPS (Sigma-Aldrich, L2880). Green-labeled yeast microcapsule was added into the cell culture medium for 4 h to detect whether yeast microcapsule could be engulfed by macrophages.

Functional detection of *IL-1β* shRNA recombinant yeast in macrophages *in vitro*

RAW264 macrophages were cultured for 24 h in DMEM (ATCC: 30-2002) containing 10% FBS supplemented with 100 ng/mL LPS (Sigma-Aldrich, L2630). Then, *IL-1β* shRNA recombinant yeast (10^5/6-well) were added into the cell culture medium for *IL-1β* shRNA recombinant yeast functional detection. After 24 h incubated at 37°C under 5% CO_2, cells were harvested by washing with PBS before cell lysis for total RNA isolation. Gene expression was detected by real-time PCR method.

Distribution of yeast in joint injury after oral administration

In order to detect whether yeast can transfer to the joint injury site after oral administration, we labeled yeast with near-infrared fluorescein Dir to get rYeast. In brief, near-infrared fluorescent dye Dir (AAT Bioquest, 22070) was used to label yeast into rYeast, which emits red light at near-infrared wavelengths. More detailed steps can be found in the Supplemental materials and methods. The articular cartilage of mouse knee was severely damaged by surgical blade. Two days after the operation recovery, mice were given 30 mg/kg rYeast or yeast every day. After continuous administration 3 times, the knee joints of mice were collected for fluorescence imaging detection using the Odyssey CLX imaging system.

Mice

Male C57BL/6 mice aged 8 weeks were purchased from the Animal Breeding and Research Centre of Xi'an Jiaotong University, PR China. They were housed under standard conditions of room temperature and dark-light cycles with plenty of food and water. All mice were treated according to the policy and regulations of the Institutional Animal Care and Use Committee. The PTOA model mouse was generated by surgical destabilization of the medial meniscus on the knee joint.[47] One month after surgery, mice were randomly allocated to four groups (n = 12). One group was treated with yeast containing pIN27-hU6-miR30 with negative control shRNA interference segment (control group). Three experimental groups were treated with yeast harboring vector pIN27-hU6- *IL1β*-shRNA. They were all given 30 mg/kg yeast microcapsule every other day. After 40 days, serum samples were collected from the tail veins for cytokine analysis via an enzyme linked immunosorbent assay (ELISA). Macrophages were isolated from the small intestine and bone marrow. Samples of the small intestine and knee joints were collected for histological analysis. RNA was extracted from intestine macrophages, bone marrow macrophages, and articular cartilage for detection of OA-related gene expression.

Tissue macrophage isolation and gene expression quantification

For macrophage sorting, small intestine digestion was performed using the method we described previously.[18] After cutting the tissue into 1- to 2-mm^3-size pieces, 25 mL collagenase IV solution was added for incubation at 37°C, 155 rpm for 60 min. Digested cells were filtered through a 70-μm sterile filter, and the cell suspension was washed twice with PBS. Bone marrow was washed to obtain cell suspension. After adding 400 μL/10^8 cells buffer (PBS with 0.5% BSA, 2 mM EDTA), CD11b microBeads (cat. no. 130-049-601, MiltenyiBiotec, Germany) were used for sorting macrophages according to the manufacturer's instructions. Cell suspension was used for total RNA extraction. OA-related gene expression was detected by real-time PCR (primers are presented in Table 2).

Table 2. The sequences of primers

Primer name	Sequence (5′ to 3′)
shDec-F	AGAATCGTTGCCTGCACATC
shDec-R	GAGATAGCAAGGTATTCAG
IL-1β-F	AACCTGCTGGTGTGTGACGTTC
IL-1β-R	CAGCACGAGGCTTTTTTGTTGT
TNF-α-F	ACCCTCACACTCAGATCATCTTC
TNF-α-R	TGGTGGTTTGCTACGACGT
Arginase-1-F	CTCCAAGCCAAAGTCCTTAGAG
Arginase-1-R	AGGAGCTGTCATTAGGGACATC
18S-F	CGGCTACCACATCCAAGGAA
18S-R	GCTGGAATTACCGCGGCT
Nr1D2-F	AGTGGCATGGTTCTACTGTGT
Nr1D2-R	GCTCCTCCGAAAGAAACCCTT
Col II-F	AGGACCTGGTAGTGCGAGTGAC
Col II-R	CTGGGCGATAGTGGAATACAACT
Col X-F	GATGCCGCTTGTCAGTGCT
Col X-R	CCAGATCTTGGGTCGTAATGC
MMP13-F	GCCCCTTCCCTATGGTGATG
MMP13-R	TTGCATTTCTCGGAGCCTGT

Western blot

Proteins were separated on 12% SDS-PAGE acrylamide gels and transferred onto a polyvinylidene fluoride (PVDF) membrane (cat. no. 10600023, GE Healthcare, Germany). The membrane was incubated with 5% skim milk and washed six times with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% [v/v] Tween 20). After incubation with primary rabbit polyclonal antibody against IL-1β (cat. no. Ab9722, Abcam, Hong Kong, PR China), the membrane was washed with TBST six times. The secondary antibody IRDye 800CW goat anti-rabbit immunoglobulin G (IgG) (H+L) was added (cat. no. 926-32211, LI-COR, USA). After washing the membrane with TBST, the signal was detected by the Odyssey CLX imaging system (LI-COR, Lincoln, NE, USA).

ELISA assay

Serum samples were harvested from the tail veins. ELISA was used to determine serum concentrations of IL-1β, IL-10, IL-12, IL-21, TNF-α, and GM-CSF using PeproTech's full ELISA kits (PeproTech, USA) according to the manufacturer's instructions. The signal was detected by Thermo Fisher Varioskan flash (Thermo, Waltham, MA, USA) (n = 6).

Histological analysis

Mouse knee joints were collected for histological analysis. Safranin O/Fast Green and H&E staining were performed. The slides were blindly graded for articular cartilage degeneration using OARSI criteria.[48] The osteoarthritis damage score was blindly assessed by three professional observers. Immunofluorescence was used to detect IL-1β (or TNF-α) expression in the small intestine and joint tissue. Immunohistochemistry was used to detect the expression of MMP13 in joint tissue.

Statistical analysis

All statistical results were presented as means ± SD. Results were analyzed using Prism version 7 (GraphPad Software) for Windows. An unpaired two-tailed Student's t test was used for comparison between two groups. Analysis of variance (ANOVA) was used to test for differences among groups. A p value of <0.05 was considered statistically significant.

The authors declare that all data supporting the finding of this study are available within the paper and its Supplemental information.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.11.006.

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AUTHOR CONTRIBUTIONS

L.Zhang, H.Peng, M.Feng, W.Zhang, and Y.K. Li, performed the experiments. All authors discussed the data. L.Zhang designed and drafted the work and final approval of the version published. All authors gave final approval of the published version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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