Activity of fibroblast-like synoviocytes in rheumatoid arthritis was impaired by dickkopf-1 targeting siRNA

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Abstract
Background: Fibroblast-like synoviocytes (FLSs), resident mesenchymal cells of synovial joints, play an important role in the pathogenesis of rheumatoid arthritis (RA). Dickkopf-1 (DKK-1) has been proposed to be a master regulator of bone remodeling in inflammatory arthritis. Here, potential impairments on the activity of FLSs derived from RA on small interfering RNAs (siRNAs) targeting DKK-1 was investigated.

Methods: siRNAs targeting DKK-1 were transfected into FLSs of patients with RA. Interleukin (IL)-1β, IL-6, IL-8, matrix metalloproteinase (MMP) 2, MMP3, MMP9, transforming growth factor (TGF)-β1, TGF-β2 and monocyte chemoattractant protein (MCP)-1 levels in the cell culture supernatant were detected by enzyme-linked immunosorbent assay (ELISA). Invasion assay and 3H incorporation assay were utilized to investigate the effects of siRNAs targeting DKK-1 on FLSs invasion and cell proliferation, respectively. Western blotting was performed to analyze the expression of nuclear factor (NF)-κB, interleukin-1 receptor-associated kinase (IRAK)1, extracellular regulated protein kinases (ERK)1, Jun N-terminal kinase (JNK) and β-catenin in FLSs.

Results: DKK-1 targeting siRNAs inhibited the expression of DKK-1 in FLSs (P < 0.01). siRNAs induced a significant reduction of the levels of IL-6, IL-8, MMP2, MMP3 and MMP9 in FLSs compared to the control group (P < 0.05). DKK-1 targeting siRNAs inhibited the proliferation and invasion of FLSs (P < 0.05). Important molecules of pro-inflammatory signaling in FLSs, including IRAK1 and ERK1, were decreased by the inhibition of DKK-1 in FLSs. In contrast, β-catenin, a pivotal downstream molecule of the Wnt signaling pathway was increased.

Conclusions: By inhibiting DKK-1, we were able to inhibit the proliferation, invasion and pro-inflammatory cytokine secretion of FLSs derived from RA, which was mediated by the ERK or the IRAK-1 signaling pathway. These data indicate the application of DKK-1 silencing could be a potential therapeutic approach to RA.

Keywords: Dickkopf-1; Fibroblast-like synoviocytes; Rheumatoid arthritis; small interfering RNAs

Introduction
Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease that primarily affects the peripheral joints and leads to articular destruction and functional disability.[1] Chronic synovitis is the main characteristic of RA and is defined by the proliferation of synovial lining cells, an important source of chemokines and cytokines associated with inflammation.[2] Inflammation in the cartilage, synovial tissue and synovial fluid can result in a pathological tissue response that destroys articular cartilage and bone tissues.[3,4] Fibroblast-like synoviocytes (FLSs), resident mesenchymal cells of synovial joints, play an important role in the pathogenesis of RA. Activation of FLSs in the setting of RA leads to a broad array of cell surface and soluble mediators which help to recruit, retain, and activate cells of immune system and resident joint.[4] As a result, activated immune cells lead to the dysregulated expression of inflammatory cytokines. Cytokines work within a complex regulatory network in RA, signaling through different intracellular kinase pathways to modulate the recruitment, activation and function of immune cells and other leukocytes.[5,6]
The Wnt signaling pathway is the key regulator of joint remodeling. Dickkopf-1 (DKK-1) is an endogenous secreted factor that acts as an antagonist of the Wnt pathway by binding the Wnt co-receptor LRP5/6. DKK-1 is secreted by FLSs in response to inflammation and has been proposed to be a master regulator of bone remodeling in inflammatory arthritis. In a previous study, we found that serum levels of DKK-1 were significantly increased in patients with RA compared to healthy controls or individuals with other rheumatic diseases such as osteoarthritis and ankylosing spondylitis. As an important mediator of bone remodeling, DKK-1 is associated with bone erosion and inflammation in RA. It has been reported that blockade of DKK-1 with an anti-DKK-1 antibody can reverse the bone-destructive pattern in experimental arthritis and mediate a bone-forming pattern. However, it is unknown whether silencing DKK-1 with small interfering RNAs (siRNAs) has the same effect as using an anti-DKK-1 antibody. We hypothesized that DKK-1 may serve as a therapeutic target in RA. In this study, we investigated the effect of silencing DKK-1 by si RNAs in the FLSs of RA.

Methods

**Ethical approval**

This study was approved by the Ethical Committee of the Peking University People’s Hospital (No. FWA00001385). All patients signed a written informed consent.

**Isolation and culture of FLSs from RA patients**

Synovial tissues were obtained from patients with RA (n = 10, female, aged 30–60 years) during knee replacement surgery. All RA patients met the criteria of the American College of Rheumatology for the classification of RA.

The synovial tissues were washed twice with phosphate-buffered saline (PBS), finely minced in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, USA) which contained 1 mg/mL collagenase (Sigma, St. Louis, MO, USA), and then incubated at 37°C for 1 h. The cell suspension was centrifuged and washed twice with PBS. Synovial fibroblasts were cultured in a 5% CO2 humidified incubator at 37°C in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Passaged cells maintained exponential growth. Since FLSs have strong adherence, the macrophage-like cells and impurities were removed by removing non-adherent cells, and the FLSs were thus purified through this passage.

**Transfection of the siRNAs in FLSs**

One day before transfection, FLSs (5 x 10^4/well) were seeded in 12-well plates (Corning, Netherlands, Dutch) with 60% to 70% confluence using complete medium under standard conditions. Cells were transfected with siRNAs targeting DKK-1 by Lipofecatamine™ 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h. The 20 ng/mL of anti-DKK-1 monoclonal antibody was used as a supposed positive control to neutralize DKK-1 secreted from FLSs. A scrambled control served as a negative control (GenePharma Co, Shanghai, China). Table 1 shows six siRNA duplexes targeting the DKK-1 gene (D1–D6) which were synthesized by chemical method (GenePharma Co, Shanghai, China). The expression levels of DKK-1 in FLSs were detected by real-time quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), and the siRNAs with the most effective inhibitory activity were selected.

### Evaluation of siRNAs in FLSs

To determine mRNA expression levels of DKK-1 in FLSs after siRNA transfection, total RNA was extracted using the TRizol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). The extracted RNA was used to synthesize complementary DNA (cDNA) in the presence of M-MLV reverse transcriptase (Promega, Madison, WI, USA). The fluorescence quantitative PCR reaction (using SYBR green, ABI) was performed on the ABI PRISM 7300 Sequence detection system (Perkin-Elmer Applied Biosystems). Primer specific sequences were: GAPDH forward: 5'-AATGAAGGGGTATCTTAGG-3'; GAPDH reverse: 5'-AAGGTGAAGGTTCGAAGTCAA-3'; DKK-1 forward: 5'-TTTCTCTAAATTTCTCCAG-3'; DKK-1 reverse: 5'- ATCCGTCAGCTATGTGCT-3' (SBs Genetech Co, Beijing, China). Individual samples were run in duplicate. After computing the relative expression of target genes, the final relative mRNA quantities of DKK-1 were represented as the ratio of DKK-1 to GAPDH. The levels of DKK-1 in the supernatant of FLSs were examined by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) in PBS overnight at room temperature. The plates were washed with PBS/Tween and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Diluted patient serum samples were added to the plates and incubated for 2 h. After washing the plates in PBS, a goat anti-human detection antibody (R&D Systems, Minneapolis, MN, USA) was added. The plates were

| Groups     | Sequence of siRNAs |
|------------|---------------------|
| DKK-1.1(D1)| 5'-GGUUUCUAAUAUACACGCUUUGTTD-3' |
| DKK-1.2(D2)| 5'-AGGCUUGAUAUGACGUUAACDDTD-3' |
| DKK-1.3(D3)| 5'-GCUCUCUUCACGGCAGCAAGAATGTTD-3' |
| DKK-1.4(D4)| 5'-UGGACCCAGAGUGUACAGCCTTDDTD-3' |
| DKK-1.5(D5)| 5'-UCGUAACCCUGUUCUCUGAAADTD-3' |
| DKK-1.6(D6)| 5'-UGGACCCAGAGUGUACAGCCTTDDTD-3' |

siRNAs: Small interfering RNAs; DKK-1: Dickkopf-1.
consequently incubated for another 2 h, and Streptavidin–
horseradish peroxidase (HRP) was added and incubated
for 20 min. After washing the plates, the substrate reagent (R&D
Systems, Minneapolis, MN, USA) was added and incubated
for another 20 min. Then, 1 mol/L sulfuric acid was added to
stop the substrate reaction, and the extinction was measured
at a wavelength of 450 nm using a multiplate ELISA reader
(Anthos Microsystems, Krefeld, Germany).

**FLSs proliferation assays**

FLSs were seeded into 96-well plates (Corning) at a density
of 6 \times 10^3 cells/well in a total volume of 0.2 mL RPMI
1640 supplemented with 10% FBS. Each culture was
performed in triplicate. After 24 hours, when the cytotoxicity
rate reached 60% to 80%, transfection with siRNAs was
performed as previously described. 10 h before the end of
culture, 1 \muCi of \[^3H\] thymidine (NEN Life Science
Products, Boston, MA, USA) was added to each well and
cells were further incubated for additional 12 h. Then, cells
were washed three times in chilled PBS to remove nonincorporated \[^3H\] thymidine. Cells were consequently
harvested onto nitrocellulose and added to scintillation fluid. The radioactivity of the incorporated \[^3H\] thymidine
was counted in a scintillation counter. FLSs proliferation
was represented as the incorporated radioactivity in counts
per minute (c.p.m).

**Cytokine quantification**

Quantitative analysis of IL-1\(\beta\), IL-6, IL-8, matrix metal-
loproteinase (MMP) 2, MMP3, MMP9, TGF-\(\beta\)1, TGF-\(\beta\)2
and monocyte chemoattractant protein (MCP)-1 levels in
the cell culture supernatant was performed by ELISA using
commercially available kits (R&D Systems, Minneapolis,
MN, USA). First, assay diluent was added to each well with
the standard samples and wells were covered with the
adhesive strip provided and incubated at room tempera-
ture. Then, each well was aspirated and washed as the
complete removal of liquid at each step is essential for good
performance. Conjugate was added to each well and the
above steps were repeated. Lastly, plates were washed with
wash buffer, and substrate solution was added to each well
and incubated for 20 to 30 min at room temperature. It
should be noted that the procedure requires the samples be
protected from light. Once 1 mol/L sulfuric acid added, the
substrate reaction was stopped, and the extinction was
measured at a wavelength of 450 nm using a multiplate
ELISA reader.

**Invasion assay**

CytoSelect™ 24-Well Cell Invasion Assay (Cell Biolabs,
San Diego, CA, USA) was used to observe the cell invasion
capacity. Under sterile conditions, a cell suspension
containing 0.5 \times 10^6 cells/mL in serum free media was
employed. The 500 \muL of media containing 10% FBS was
added to the lower well of the invasion plate, and 300 \muL
of the cell suspension solution was added to the inside of
each insert. After incubation for 48 h at 37°C in a 5% CO\(\text{2}
\) atmosphere, the media was carefully aspirated from the
inside of the insert. Then, two to three wet cotton-tipped
swabs were used to gently swab the interior of the inserts to
remove non-invasive cells. After that, the insert was
transferred to a clean well containing 400 \muL of cell stain
solution and incubated for 10 min at room temperature.
The stained inserts were gently washed several times in a
beaker of water and allowed to air dry. Then, 200 \muL of
extraction solution was added per well and each insert was
transferred to an empty well and incubated for 10 min on
an orbital shaker. Lastly, 100 \muL from each sample was
transferred to a 96-well microtiter plate and the absorb-
bance (A) 560 nm was measured in a plate reader.

**Western blotting analysis**

Western blotting was performed according to standard
procedures. A \(\beta\)-actin signal was used as an internal
control. Cell lysates were separated on a 10% SDS/PAGE
gel and transferred onto polyvinylidene fluoride mem-
branes. Antibodies against nuclear factor (NF)-\(\kappa\)B, tumor
necrosis factor (TNF) receptor associated factor \(\beta\)
(TRAF6), interleukin-1 receptor-associated kinase (IRAK)1,
extracellular regulated protein kinases (ERK)1, Jun N-terminal
kinase (JNK)1/2, p38a/\(\gamma\), \(\beta\)-catenin and
\(\beta\)-actin (R&D Systems, Minneapolis, MN, USA) were
used, and secondary HRP-conjugated anti-rabbit or
anti-mouse IgG antibodies (R&D Systems, Minneapolis,
MN, USA) were added. The signals were visualized by
using an ECL detection kit (PerkinElmer, Boston,
MA, USA).

**Statistical analyses**

All statistical analyses were performed in SPSS 24.0 (SPSS
Inc., Chicago, IL, USA). Results were conformed to the
normal distribution and expressed as mean \pm
standard deviation (SD). Statistical analysis was done with the
Student’s \(t\) test for comparison of two groups and one-way
analysis of variance (ANOVA) for multiple comparisons.
A \(P\) value <0.05 was considered statistically significant.

**Results**

**siRNAs targeting DKK-1 downregulated the expression of
DKK-1 in RA FLSs**

In order to screen the siRNAs with the strongest inhibitory
effect, six siRNA duplexes targeting DKK-1 (D1–D6) were
synthesized. As shown in Figure 1, all of the six pairs of
DKK-1 specific siRNAs significantly inhibited the expres-
sion of DKK-1 regardless of the mRNA level or protein
level as compared with the expression observed in the
control \(P < 0.01\). Among them, the two most effective
siRNAs were selected for the subsequent experiments (D2
and D6).

**siRNAs targeting DKK-1 inhibited RA FLSs proliferation**

After 48 h of transfection, proliferation of RA FLSs was
determined by \[^3H\] thymidine incorporation. The FLSs
isolated from RA patients responded positively to TNF-\(\alpha\)
(20 ng/mL) stimulation, and the c.p.m of the control FLSs
increased from 6876 \pm 1340 to 10,174 \pm 1228, \(P < 0.05\).
Down-regulation of DKK-1 by siRNAs significantly
inhibited FLSs proliferation both with and without TNF-stimulation \( (P < 0.01, \text{Figure } 2) \). However, the anti-DKK-1 monoclonal antibody did not show the same effect as the one observed with siRNAs.

**siRNAs targeting DKK-1 suppressed the inflammatory response of FLSs from RA patients**

Infiltration and migration of inflammatory cells into the synovium play an important role in RA pathogenesis. Several mediators are involved in this process; thus, our study investigated whether DKK-1 plays a role in this process by regulating the production of pro-inflammatory mediators of FLSs. TNF-\( \alpha \) stimulation led FLSs to produce several cytokines, including IL-6 and IL-8 (Figure 3, shown in the control group with TNF-\( \alpha \) stimulation) compared to the control group lacking TNF-\( \alpha \) stimulation. However, siRNAs inhibited the secretion of IL-6 and IL-8 in FLSs both with and without TNF-\( \alpha \) stimulation \( (P < 0.05 \text{ and } P < 0.01, \text{respectively}) \). The expression of TGF-\( \beta 2 \) and MCP-1 in FLSs was not changed by treatment with siRNAs (data not shown).

**siRNAs targeting DKK-1 suppressed the invasive behavior and MMPs expression of RA FLSs**

The invasive property of RA patient-derived FLSs has been shown to correlate with disease severity and radiographic damage. The MMPs are key mediators of the invasive phenotype of FLSs. Therefore, we further investigated the effect of siRNAs on invasive behavior and MMPs secretion of FLSs. As shown in Figure 3, both the invasive behavior of RA FLSs and production of MMPs in the control group with TNF-\( \alpha \) were significantly increased compared to the control without TNF-\( \alpha \) stimulation. Unexpectedly, siRNAs significantly inhibited the invasive behavior of FLSs \( (0.19 \pm 0.04 \text{ for D2, } 0.19 \pm 0.06 \text{ for D6 vs. } 0.27 \pm 0.04, P < 0.05) \) both without TNF-\( \alpha \) stimulation and with it \( (0.24 \pm 0.02 \text{ for D2, } 0.23 \pm 0.05 \text{ for D6 vs. } 0.34 \pm 0.04, P < 0.05) \) [Figure 4].
Consistently, the production of MMP2, MMP3 and MMP9 was significantly down-regulated by siRNAs with or without TNF-α stimulation [Figure 3]. While an anti-DKK-1 antibody could inhibit the production of MMP3, it had no effect on MMP2 and MMP9. Osteoprotegerin (OPG), which blocks receptor activator of NF-κB Ligand (RANKL)-induced osteoclastogenesis and results in the inhibition of bone resorption, was found to be increased by siRNAs without TNF-α stimulation ($P < 0.05$).

Figure 3: siRNAs targeting DKK-1 suppressed the secretion of cytokines in FLSs from rheumatoid arthritis patients. Compared with the control, siRNAs (D2, D6) suppressed the secretion of MMP2 (A), MMP3 (B), MMP9 (C), IL-6 (D), and IL-8 (E) in rheumatoid arthritis FLSs. siRNAs promoted the production of OPG (F). $^* P < 0.01$, $^† P < 0.05$. siRNAs: Small interfering RNAs; DKK-1: Dickkopf-1; FLSs: Fibroblast-like synoviocytes; MMP: Matrix metalloproteinase; IL: Interleukin; OPG: Osteoprotegerin.
siRNAs targeting DKK-1 inhibited the MAPK and IRAK1, but had no effect on NF-κB pathway

To determine the intracellular mechanisms involved in cell activation, we explored the effect of DKK-1 on signaling pathways that induce cell activation. We found that siRNA (D2) targeting DKK-1 could inhibit the expression of IRAK1 and ERK1. In addition, β-catenin, a pivotal downstream molecule of the Wnt signaling pathway was up-regulated by D2. A DKK-1 monoclonal antibody significantly increased the expression of β-catenin but it had no effect on the expression of IRAK1 and ERK1 [Figure 5].

Discussion

Our results present a novel account of in vitro RNAi-based anti-inflammatory effects. RNAi is a useful and simple tool to better understand the pathophysiology of RA and evaluate new therapeutic candidates. Importantly, siRNAs targeting DKK-1 demonstrated potent anti-inflammatory effects and alleviated the invasive behavior of FLSs, which is promising for RA treatment.

TNF-α is a major pro-inflammatory factor involved in the pathogenesis of RA. We mimicked the inflammatory environment of RA in vitro with TNF-α.[17,18] In our study, we showed that TNF-α could stimulate FLSs to produce IL-6, IL-8, and MMPs, and promote proliferation and invasion of FLSs. siRNAs could inhibit the activity of FLSs with or without the stimulation of TNF-α.

In a previous study, Diarra et al.[11] reported that the blockade of DKK-1 with an anti-DKK-1 antibody could reverse the bone-destructive pattern of a mouse model of RA and restore a bone-forming pattern. In this study, we found that siRNAs targeting DKK-1 had profound inhibitory effects on the FLSs of RA, but did not have the same effect as what an anti-DKK-1 antibody did. Notably, in some characteristics assessed, silencing DKK-1 had a stronger effect. Astrup et al.[19] found that silencing DKK-1 attenuated the release of IL-6 and IL-8 in Rickettsia conorii-exposed human umbilical vein endothelial cells, which was found to be consistent with our results. Moreover, Rickettsia conorii affects DKK-1 and inflammatory cytokines differently in in vivo and in vitro conditions in endothelial cells. This suggests that an anti-DKK-1 antibody and siRNAs targeting DKK-1 do not exert inhibitory effects in the same manner in different conditions.

It is well-known that there is an interaction between DKK-1 and key molecules of joint degradation, such as those in the RANKL-OPG system.[20] In a physiological state, there exists a balance between cortical bone formation and resorption next to the joints.[21] RA leads to an imbalance in bone formation and resorption. Bone formation is hampered by the over-expression of DKK-1, which suppresses Wnt and OPG signals, whereas bone resorption...
is enhanced.\textsuperscript{[22]} OPG elevation by siRNAs confirms that DKK-1 may be involved in the process of bone destruction and siRNAs targeting DKK-1 may reverse or slow down this process.

Many cytokines, chemokines, and growth factors which stimulate FLSs migration and the synthesis of numerous MMPs and cathepsins are hypothesized to be important for cartilage matrix breakdown. Our results show that reduced expression of DKK-1 in FLSs can decrease the production of MMPs and inhibit the invasive behavior of FLSs. Thus, siRNAs targeting DKK-1 may directly block the bone destruction of RA.

Another major consequence of RA FLSs activation is the synthesis of a broad array of soluble and cell surface pro-inflammatory molecules with many diverse functions through their activation of multiple signal transduction pathways, including NF-kB and mitogen-activated protein kinase (MAPK).\textsuperscript{[23]} Among the MAPK families, ERK, JNK and p38, have been implicated in RA.\textsuperscript{[24]} JNK and ERK play very important roles in extracellular matrix turnover and are activated in the RA synovium. They regulate MMP gene expression in cultured FLSs and mediate joint destruction in rat adjuvant arthritis.\textsuperscript{[25,26]} DKK-1 siRNAs can significantly decrease the expression of ERK and MMPs in FLSs suggesting that siRNAs targeting DKK-1 may improve FLSs features through the MAPK pathway.

Previous studies have demonstrated that the IL-1 receptor associated kinase 1 (IRAK-1) suppresses IL-6/IL-8 secretion.\textsuperscript{[27,28]} Physiological studies have shown that Irak1-null mice are protected from various inflammatory diseases including experimental autoimmune encephalomyelitis and atherosclerosis, though the mechanism for this remains unknown. In this study, we found that siRNAs targeting DKK-1 down-regulated the secretion of IL-6 and IL-8, that may be mediated by the repression of IRAK-1 signaling.

Proteins synthesized by Wnt genes are key mediators of osteoblastogenesis and govern the formation of the skeleton during the development of the embryo.\textsuperscript{[29]} Several members of the Wnt protein family bind a receptor complex consisting of LPR5/6 and frizzled receptors on the plasma membrane of mesenchymal cells, which signals osteoblast differentiation by engaging the intracellular protein β-catenin. DKK-1 functions as a natural inhibitor of the Wnt signaling.\textsuperscript{[30]} In this study, the interference of DKK-1 with either siRNAs or an antibody targeting DKK-1 may increase the expression of β-catenin.

The limitations of this study as are follows: in vivo experiments could not be performed due to technical difficulties. The use of siRNA for in vivo applications remains difficult as optimizing the intracellular delivery of the siRNA and determining the stability of the compound pose complications. Ideally, these experiments may be repeated in vivo once some of these complications have been resolved.

In this study, siRNAs targeting DKK-1 exerted a profound inhibitory effect on the proliferation, invasive behavior, and inflammatory responses of FLSs, which was mediated by the ERK or the IRAK-1 signaling pathway. These data suggest that DKK-1 silencing represents a potential therapeutic approach for RA.

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**Conflicts of interest**

The authors declare that they have no conflicts of interest.

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