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Efficient inhibition of the formation of joint adhesions by ERK2 small interfering RNAs

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ABSTRACT

Transforming growth factor-β1 and fibroblast growth factor-2 play very important roles in fibroblast proliferation and collagen expression. These processes lead to the formation of joint adhesions through the SMAD and MAPK pathways, in which extracellular signal-regulated kinase (ERK)2 is considered to be crucial. Based on these theories, we examined the effects of a lentivirus-mediated small interfering RNA (siRNA) targeting ERK2 on the suppression of joint adhesion formation in vivo. The effects were assessed in vivo from different aspects including the adhesion score, histology and joint contracture angle. We found that the adhesions in the ERK2 siRNA group became soft and weak, and were easily stretched. Accordingly, the flexion contracture angles in the ERK2 siRNA group were also reduced ($P < 0.05$ compared with the control group). The animals appeared healthy, with no signs of impaired wound healing. In conclusion, local delivery of a lentivirus-mediated siRNA targeting ERK2 can ameliorate joint adhesion formation effectively and safely.

Introduction

The formation of fibrous joint adhesions following surgery or trauma severely restricts functional recovery, and is a major problem in the field of orthopedics. Some pharmaceutical agents have been examined for their efficacy in preventing adhesions after local administration [1–4]. Although these attempts have achieved limited success, adhesion formation generally remains an unsolved problem because the pharmaceutical agents need to be administered into the articular cavity frequently or continuously, and this is inconvenient for clinical applications. Therefore, a new strategy for the treatment of joint adhesions is in urgent demand.

RNA interference (RNAi) is a process by which double-stranded RNA triggers the destruction of mRNAs sharing the same sequence. It is initiated by small interfering RNAs (siRNAs), comprising short duplexes of 19–23-nucleotide single-stranded RNAs, which direct the degradation of the target RNAs [5–7]. Based on their advantages of significant and long-lasting inhibitory effects, siRNAs have been demonstrated to be of great value for the treatment of many joint diseases, including osteoarthritis, rheumatoid arthritis and periprosthetic osteolysis [8–10]. Regrettably, however, the aspect of whether siRNAs can be used to prevent joint adhesion formation has not been investigated. Previously, we demonstrated that extracellular signal-regulated kinase (ERK)2 plays crucial roles in suppressing the collagen expression and proliferation of rat joint adhesion tissue fibroblasts (RJATFs) stimulated by transforming growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2 [11]. Since adhesions are considered to result from fibroblast proliferation and collagen expression, this study aimed to achieve marked inhibition of joint adhesion formation by intra-articular administration of a lentiviral-mediated ERK2 siRNA.

Materials and methods

Lentiviral vector construction, virus production and transfection. The pshRNA-H1-Luc lentivector (System Biosciences) used in this study was designed to coexpress luciferase cloned from the copepod. The sequence of the siRNA targeting rat ERK2, 5′-GCACCT CAGCAATGATCAT-3′, has previously been proven to efficiently downregulate rat ERK2 [11]. A missense siRNA (MS siRNA) with the sequence 5′-TGCAGTTCGGAATCAGCTT-3′ was designed as a control. Pairs of complementary oligonucleotides containing these sequences were synthesized (Invitrogen) and cloned into the pshRNA-H1-Luc lentivector. 293T producer cells were cotransfected with pPACK Packaging Plasmid Mix (System Biosciences) and the pshRNA-H1-Luc lentivectors containing the shRNA sequences using Lipofectamine™ 2000 (Invitrogen). Viral supernatants were harvested after 48 h, and the titers were determined with serial dilutions of concentrated lentivirus.

Animal model. Thirty female Lewis rats aged 12 weeks were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences). All experimental procedures were approved by the authors' Institutional Animal Review Committee. The rats

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were randomly assigned to one of three groups (n = 10 each): ERK2 siRNA group; MS siRNA group; control group. Under inhalation anesthesia (2–3% isoflurane and oxygen), the right knee joint was prepared for surgery under aseptic conditions. After a skin incision, the knee was opened through a lateral parapatellar approach and the medial and lateral sides of the femoral condyle were exposed. A partial capsulotomy and synovectomy were performed using an osteotome and an osteochondral portion of the condyle (approximately 4 × 2 mm) was removed until the underlying cancellous bone was exposed (Sup. 1A). Each operated limb underwent knee joint immobilization at 140° of flexion with a Kirschner wire for 30 days (Sup. 1B). In the ERK2 siRNA group, 0.1 ml of culture medium containing the lentivirus-mediated ERK2 siRNA was injected into the articular cavity after incision closure at days 3 and 7, respectively. Similarly, 0.1 ml of culture medium containing the lentivirus-mediated MS siRNA was applied in the MS siRNA group, and the same volume of medium without a viral vector was applied in the control group. To reduce the risk of infection, the animals were postoperatively treated with an antibiotic (Baytril; Bayer AG Leverkusen) for 7 days.

In vivo bioluminescence assay. Bioluminescence assays comprise a high-sensitivity and non-invasive technique for monitoring specific cellular and genetic activities in a living organism. At 14 and 28 days after transfection, the luciferase expression and distribution in the individual rats in the ERK2 siRNA group were measured using a Xenogen IVIS 50 Bioluminescence System (R&D Systems).

Measurement of the contracture angle. Animals were euthanized at 30 days after the surgery by inhalation anesthesia (2–3% isoflurane and oxygen), and immediately subjected to biomechanical evaluations. After careful removal of the Kirschner wire, the angle of flexion contracture was determined on a lateral view radiograph of the right knee taken under an extension torque of 4 N, and the angle "*a*" was defined as the contracture angle (Sup. 1C). All measurements were made within 15 min after euthanasia (Table 1).

Macroscopic evaluation. Following measurement of the contracture angle, each joint was exposed by a parapatellar skin incision and held at 140° of flexion. The presence and severity of intra-articular adhesions were assessed by a blinded observer using a severity score scale of 0–4: grade 0, no adhesions; grade 1, filmy weak adhesions; grade 2, mild adhesions; grade 3, moderately dense adhesions; grade 4, dense, fibrous adhesions.

Histological evaluation of adhesion tissues. After macroscopic evaluation of the adhesions, the knee joints were excised while preserving all the connective tissues involved in fibrotic adhesive scar formation. Each biopsy was fixed in 10% buffered formalin for 1 week and decalcified for 2 weeks. The tissue was embedded in paraffin, and 6-μm sections were prepared in the vertical plane to the femoral axis. The sections were stained with hematoxylin and eosin and examined microscopically.

Statistical analysis. Statistical analyses among the groups were performed by one-way analysis of variance (ANOVA) with a Student–Newman–Keuls post hoc *t*-test. Data are shown as means ± standard deviation (SD). Differences were considered to be statistically significant for values of $P < 0.05$. All statistical analyses were conducted using SPSS 11.0 (SPSS Inc.).

Table 1
Contracture angle in each group.

Group	Contracture angle (°)
The control group	76 ± 15
The MS siRNA group	76 ± 18*
The ERK siRNA group	32 ± 9**

All animals in each group were used for this evaluation. Data are represented as the means ± SE.
* $P > 0.05$ compared with the control group.
** $P < 0.05$ compared with the control group.

Results

Among the 30 rats used in this study, two died on days 14 and 20, respectively, because of infection (one in the MS siRNA group and one in the control group). These two animals were excluded from the study. The other animals gained weight and appeared healthy, with no signs of impaired wound healing.

The luciferase fluorescence detected at 14 days after transfection demonstrated that local delivery of the lentivirus-mediated ERK2 siRNA caused a localized silencing effect in the articular cavity (Sup. 2A). Moreover, the luciferase fluorescence detected after 28 days revealed similar results (Sup. 2B).

The flexion contracture angles in the control and MS siRNA groups ranged from 71° to 91° with a mean value of 82°. Although no significant differences were observed between the MS siRNA and control groups, the contracture angles in these two groups were significantly larger than those in the ERK2 siRNA group (range, 25–38°; mean value, 32°).

At 30 days after the surgery, thick fibrous adhesions developed in the knees of the rats in the control and MS siRNA groups (Figs. 1A and 2B). In contrast, the adhesions in the ERK2 siRNA group were soft and weak, and were easily stretched (Fig. 1C). The adhesion scores for the ERK2 siRNA group were significantly lower than those in the control and MS siRNA groups (data not shown).

Histologically, the adhesion tissues in the control and MS siRNA groups were dense, thick and fibrous (Fig. 2A and B), while those in the ERK2 siRNA group were loose and thin with sparse fiber formation (Fig. 2C). In all three groups, the predominant cells were considered to be fibroblasts, and few inflammatory cells were observed. There was no apparent difference in the cell densities between the control and MS siRNA groups. On the contrary, the cell density in the ERK2 siRNA group was low.

Discussion

TGF-β1 and FGF-2 play very important roles in fibroblast proliferation and collagen expression. These processes lead to the formation of joint adhesions through the SMAD and ERK pathways. TGF-β1 and FGF-2 neutralization by continuous treatment with corresponding antibodies can reduce adhesion formation [3,4]. However, these antibodies cannot be applied clinically because of the necessity for their continuous administration. TGF-β1 contributes to collagen expression by fibroblasts through the SMAD pathway, while FGF-2 stimulates fibroblast proliferation through the ERK pathway. Some previous studies have strongly indicated a synergizing role for ERK in the SMAD-mediated signaling initiated by TGF-β in some cells involved in fibrotic diseases, such as mesangial cells, human dermal fibroblasts, myofibroblasts and cardiac fibroblasts [12–15]. In addition, recent research has indicated that ERK2 plays a positive role in controlling normal and Ras-dependent cell proliferation [16]. Further studies have shown that the predominant role of ERK2 in cell proliferation is caused by its markedly higher expression compared with ERK1 [17,18]. Taken together, ERK2 is considered to be crucial for controlling fibroblast proliferation and collagen production. Based on these assumptions, lentivirus-mediated siRNAs targeting ERK2 were used to suppress the proliferation and collagen expression of RJATFs in our previous study [11]. In this study, the effects of a lentivirus-mediated ERK2 siRNA on the inhibition of joint adhesion formation were confirmed in vivo from different aspects including the adhesion score, histology and joint contracture angle. As well as inhibiting cell proliferation and collagen expression, ERK2 silencing can also abolish trophoblast development, mesoderm differentiation and T cell development [19–22]. However, unlike c-Jun N-terminal kinase/stress-activated protein kinase, which is associated with apoptosis,

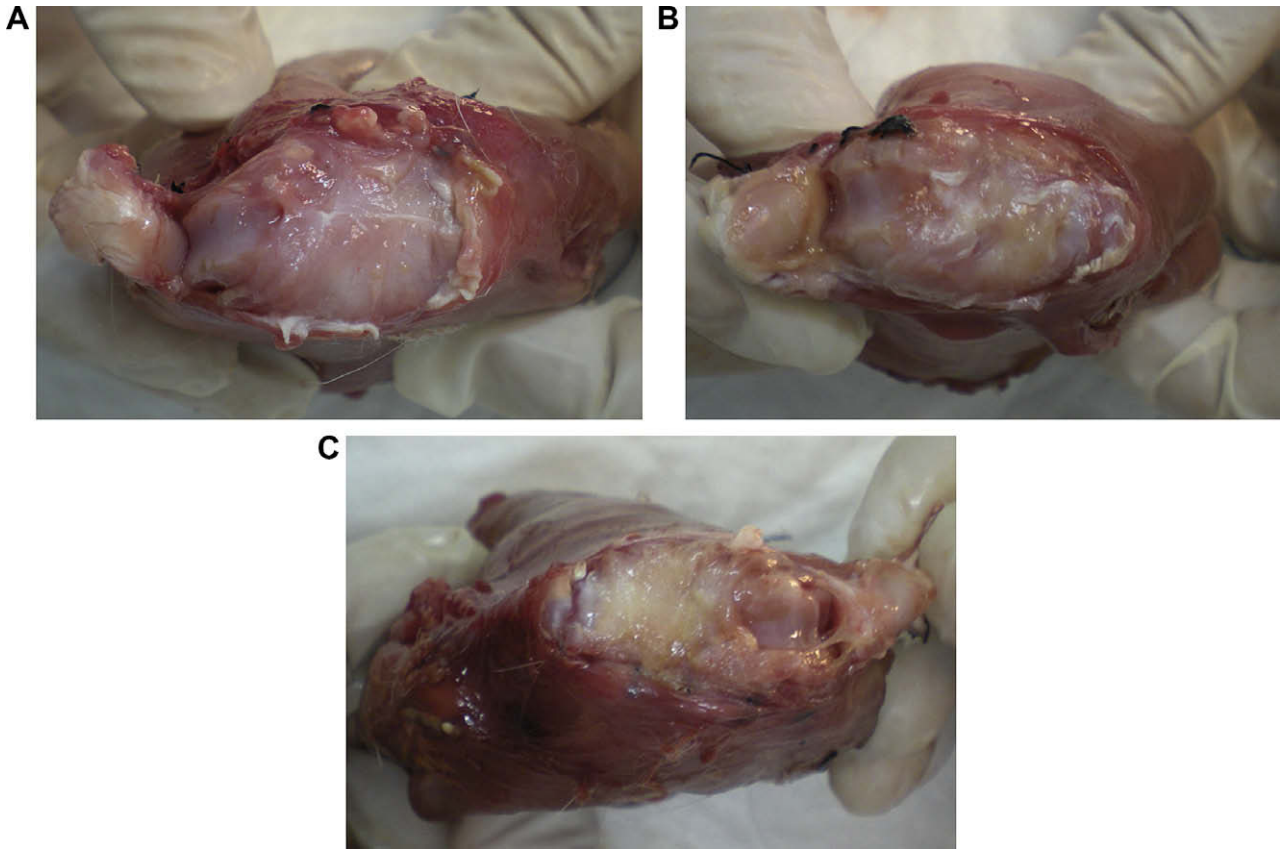

Fig. 1. Photographs of adhesions in the knee joints. (A–C) In comparison with the adhesions in the control group (A) and MS siRNA group (B), the adhesions in the ERK2 siRNA group (C) are reduced in both extent and volume.

ERK mainly responds to mitogens [23,24]. Consequently, ERK2 silencing barely influences normal chondrocytes and synovial cells in the joint cavity because these cells are static.

An animal model that can accurately mimic the features of human joint adhesions is essential for preclinical efficacy and safety evaluations of this form of gene therapy. Researchers have developed an intra-articular adhesion model in rabbits, which is currently being used to evaluate the effects of several attempts to inhibit joint adhesion formation [1]. In this study, an intra-articular adhesion model in rats was used to evaluate the effects of gene therapy for the treatment of joint adhesions. In fact, the high incidence (100%) of joint adhesions found in the control group indicated that the experimental surgical procedure used was adequate for this type of study. In addition, the adhesions in this model developed in the articular cavity, meaning that the adhesion tissues were of sufficient volume to enable biochemical analyses without contamination by other tissues.

Intravenous injection, which is the most widely used delivery method for introducing siRNAs into animals, may cause side effects through systemic downregulation of the target gene. Therefore, there is an urgent need to develop novel methods for the safe and efficacious delivery of siRNAs in vivo. In recent years, various local delivery methods for siRNAs (intravitreal, intracerebellar, intranasal and intra-air-pouch local delivery methods) have been evaluated in several studies, and have resulted in higher silencing efficacies and no systemic side effects [25–28]. Local injection of siRNAs into the murine knee joint inhibited paw inflammation effectively, while target gene silencing was not found in other organs [29]. In addition, intravitreal delivery of a siRNA every 6 weeks showed efficacy in clinical trials for macular degeneration and demonstrated a satisfactory safety profile [30]. All these studies suggest that local administration of a siRNA may be a promising strategy in the field of RNAi research. In our study, the predominant luciferase fluorescence was localized in the articular cavity without attenuation of the fluorescence. These findings indicate that the lentivirus-mediated siRNA was stably transfected only in the articular cavity and that no transfection was detected in other parts of the animals. These findings are in accordance with the above-mentioned studies, and suggest that local delivery of a lentivirus-mediated siRNA represents a safe delivery system for a siRNA.

The lentivirus-mediated gene delivery system has several advantages over other viral or non-viral gene delivery systems, such as high infection efficiency, wide variety of target cells including dividing and non-dividing cells, long-term infection owing to gene integration into the chromosome of host cells, and the absence of toxicity or immune responses [31–34]. Therefore, we selected a lentivirus as a gene delivery system for in vivo experiments. In this study, a lentivirus-mediated gene delivery system was able to efficiently deliver a ERK2 siRNA into the periarticular tissue of rabbits, and the effects were durable. Unlike antibodies, such as anti-FGF-2 or anti-TGF-β1 antibodies, ERK2 siRNAs transfected into joint adhesion tissue fibroblasts will exert lasting effects and the fewer administrations required will make ERK2 siRNAs more convenient for clinical applications.

In conclusion, we have demonstrated that local delivery of a lentivirus-mediated siRNA targeting ERK2 ameliorated joint adhesion formation effectively and safely in a rat model, which reflects the predominant role of ERK2 in joint adhesion formation. This study provides a novel and promising strategy that will serve as

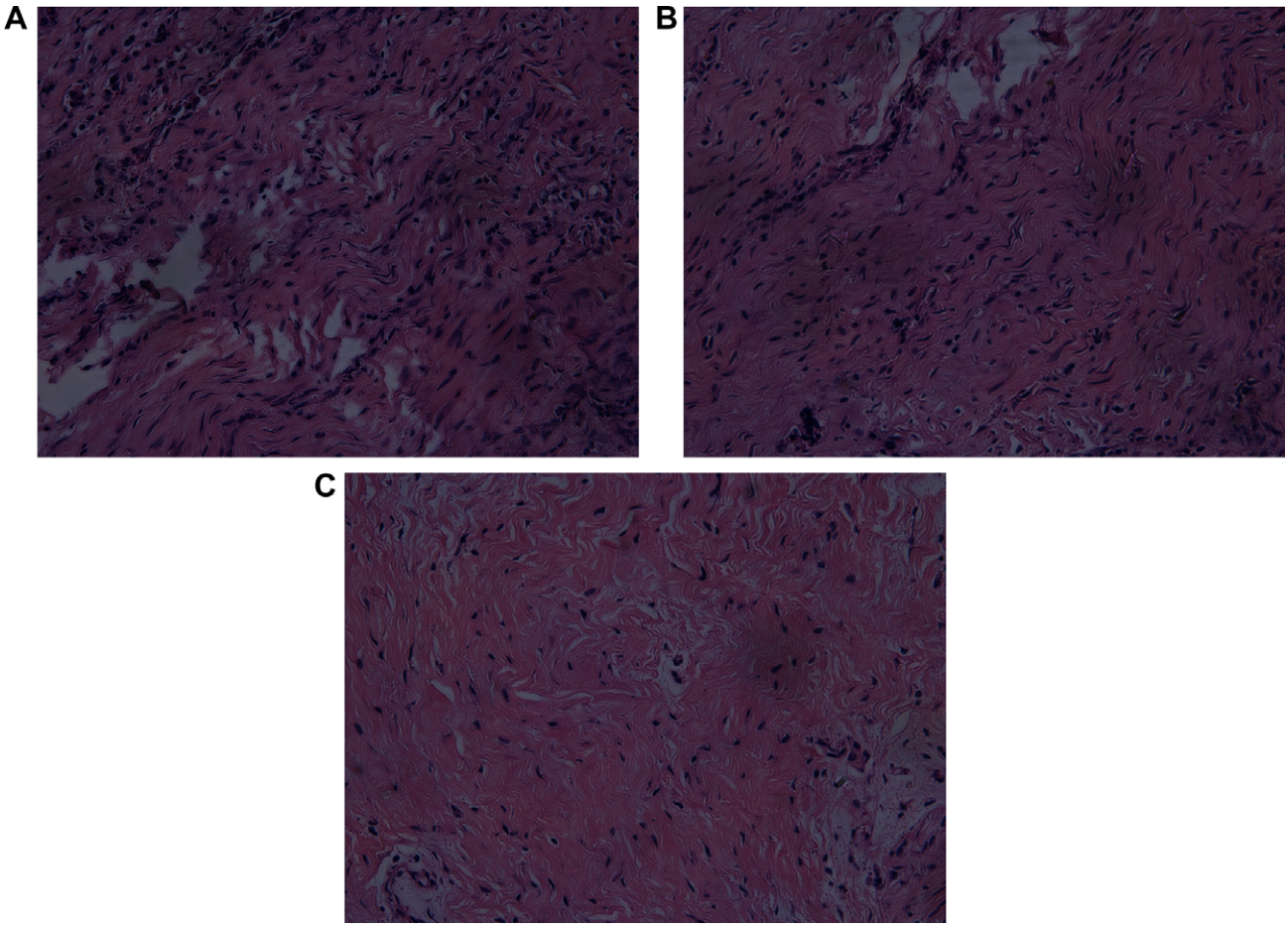

Fig. 2. Histologic analysis of adhesion tissues. (A) Control group. (B) MS siRNA group. (C) ERK2 siRNA group. The sections are stained with hematoxylin and eosin (original magnification, 100×).

an alternative for the prevention of joint adhesion formation. Further studies involving larger animals are required to support the hypothesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.140.

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