# ERK2 small interfering RNAs prevent epidural fibrosis via the efficient inhibition of collagen expression and inflammation in laminectomy rats

Chao Zhang [a], Xiaohong Kong [b], Chang Liu [b], Zhipin Liang [b], Huabing Zhao [c], Wei Tong [c], Guangzhi Ning [a], Wenyuan Shen [b], Liwei Yao [a], Shiqing Feng [a,*]

[a] Department of Orthopedics, Tianjin Medical University General Hospital, Tianjin 300052, China
[b] School of Medicine, Nankai University, Tianjin 300071, China
[c] Tianjin Key Laboratory for Prevention and Control of Occupational and Environmental Hazard, Logistics College of CAPF, Tianjin 300309, China

## ARTICLE INFO

## ABSTRACT

Laminectomy is a widely accepted treatment for lumbar disorders. Epidural Fibrosis (EF) is a common post-laminectomy or post-discectomy complication, which is thought to cause recurrent pain. RNA interference (RNAi) is a process by which double-stranded RNA triggers the destruction of mRNAs sharing the same sequence. Previously, extra-cellular signal-regulated kinase (ERK) 2 plays crucial roles in suppressing the collagen expression. To investigate the effects of lentiviral ERK2 siRNA on the prevention of post-laminectomy EF formation in a rat model, a controlled double-blinded study was conducted in 75 healthy adult Wistar rats that underwent laminectomy. They were divided randomly into 3 groups according to the treatment method: (1) control group; (2) ERK scrRNA group; (3) ERK siRNA group. All rats were euthanized humanely 4 weeks post-laminectomy. The hydroxyproline content, Rydell score, vimentin cells density, fibroblasts density, inflammatory cells density and inflammatory factors expressions were performed. The hydroxyproline content, Rydell score, vimentin cells density, fibroblasts density, inflammatory cells density and inflammatory factors expressions all suggested better results in ERK siRNA group than other two groups. None of the rats expired and no obvious adverse effects were observed. Local delivery of a lentiviral siRNA targeting ERK2 can prevent epidural scar adhesion in post-laminectomy rat via inhibiting collagen expression and inflammation.

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## 1. Introduction

Epidural fibrosis (EF) is the development of the deposition of dense scar tissue adjacent to the dura matter, which distort normal tissue architecture with leading failed back surgery syndrome (FBSS) post-laminectomy [1]. With the exhibition of a series of symptoms including significant functional disability and recurrent radicular pain, FBSS was reported to attack in 8–40% of patients who undergo lumbar disc surgery [2]. According to the previous literature, EF, leading compression or tethering the nerve roots, is one of the causative factors in FBSS [3].

Currently, different kinds of biological and non-biological agents or mechanical barriers have been administered to prevent EF formation, such as Adcon-L, anti-inflammatory agents, animal collagen membranes, Gelofoam, honey and others [4–9]. However, no satisfactory solutions for treating EF have been found to date.

Small interfering RNAs (siRNAs), comprising short duplexes of 19–23 nucleotide single-stranded RNAs, is able to lead the degradation of the target RNAs [10–12]. Therefore, siRNAs could silence the expression of specific post-transcriptional gene. With a series of advantages of significant and long-lasting inhibitory effects, siRNAs have been reported to be administrated in many orthopedics diseases, such as osteoarthritis, rheumatoid arthritis and periprosthetic osteolysis [13–15]. To date, whether gene therapy can be applied to prevent EF formation has not been reported.

Previously, extra-cellular signal-regulated kinase (ERK) 2 plays crucial roles in suppressing the collagen expression and fibroblasts proliferation [13,14]. In our rat laminectomy model, we investigated whether lentiviral ERK2 siRNA attenuated EF by regulating the expressions of interleukin 6 (IL6), transforming fac-

tor-β1 (TGF-β1) and collagen, which are reported to play an important role in the promotion and/or development of EF.

## 2. Material and methods

### 2.1. Animals

Subjects were 75 Wistar, young-adult, female rats (Radiation Study Institute-Animal Center, Tianjin, China) that weighed approximately 250 g at the time of surgery. The rats were housed in the vivarium of the Tianjin Medical University on a 12:12 h light:dark cycle with clean food and water available ad libitum. Experiments were carried out in compliance with the principles of EU Directive 2010/63/EU and were approved by the local ethical committee. All rats were randomly divided into three groups (25 rats per group): (1) control group (laminectomy with DMEM treatment); (2) ERK scrRNA group (laminectomy with lentiviral ERK2 scramble RNA treatment); (3) ERK siRNA group (laminectomy with lentiviral ERK2 siRNA treatment).

### 2.2. Lentiviral vector construction and virus packaging

The pFG12 lentiviral vector, constructed with green fluorescent protein (GFP) for detection, was used as the siRNA expression system [16]. The siRNA targeting rat ERK2 whose sequence is 5′-GCACCTCAGCAATGATCAT-3′. A scramble siRNA with the sequence 5′-TGCAGTTCGGAATCAGCTT-3′ was established as a control. Pairs of complementary oligonucleotides containing these sequences were synthesized (Invitrogen) and cloned into the pFG12 lentiviral vector. 293T producer cells were cotransfected with pFG12 and helper plasmids. Transfection was performed as described in the Invitrogen siRNA Transfection Manual using Lipofectamine 2000 (Invitrogen). Then 48 h post transfection, the cultured supernatant was harvested, and concentrated with ultracentrifugation. The lentivirus was stocked in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen). The titer of the lentiviral stocks was assessed using serial dilutions to HeLa cells. The viral titer was $4 \times 10^8$ TU/mL for lentiviral ERK2 siRNA and $5 \times 10^8$ TU/mL for lentiviral ERK2 scramble RNA control.

### 2.3. Surgery

All surgical procedures were performed under aseptic conditions. Rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g body weight, ip) and restrained on a warm pad in the prone position. Dorsal incisions were made in the skin and underlying muscles. Muscles retracted and a partial laminectomy was performed at lumbar segment 1 ($L_1$). Gauze was used for hemostasis. Close attention was paid not to traumatize the dura or the nerve roots. After the different disposes for three groups, the muscles of the incision were then closed with 5–0 absorbable braided suture, and the skin was closed. All rats were placed into an incubator for 2–3 h to recover from anesthesia and then returned to their home cages in the colony room.

To verify that the nerve roots and spinal cord were not intraoperatively injured, all rats underwent pre- and post-operative neurobehavioral assessment using the Basso, Beattie, and Bresnahan (BBB) locomotion test [17]. This scale assesses posture, weight support, and coordination during open field locomotion. Sensory-evoked potentials (SEPs) were measured in rats both pre- and post-operatively to evaluate neurological deficits.

### 2.4. Lentiviral siRNA deliver system

In the ERK siRNA treatment group, 10 μl lentiviral ERK2 siRNA was injected into the paraspinal muscle before incision closure.

Similarly, 10 μl lentivirual ERK2 scramble RNA was applied in the ERK scrRNA group, and the same volume of DMEM (serum free) was applied in the control group. To reduce the risk of infection, the rats were postoperatively given the antibiotic (Baytril; Bayer AG Leverkusen) for 7 days. The dose of lentiviral ERK2 siRNA was chosen based on previous studies [18–20].

### 2.5. Detection of virus delivery and histological analysis

GFP detecting and histological analysis were performed 4 weeks after surgery in ten rats randomly selected from each group.

Intracardial perfusion with ice-cold saline followed by 4% paraformaldehyde was performed in five rats. The entire $L_1$ vertebral column, including the paraspinal muscles and epidural scar tissue, was resected en bloc. The fixed samples were serially cryosectioned at 10 μm. The expression of GFP was directly viewed using an epifluorescent microscopy system (Leica CM3050S, Germany).

The same perfusion and samples harvest were performed in other five rats. After decalcification and dehydration with Cal-Ex II solution (Thermo Fisher Scientific, USA) for 2 days, samples were embedded in paraffin, and 5-μm axial sections of the laminectomy site were stained with hematoxylin and eosin (H&E). Epidural scar adhesion was evaluated under a light microscope. Three counting areas were selected at the center and the margins of the laminectomy sites. The numbers of fibroblasts were calculated as follows: cells in the three different areas were counted, and the mean was calculated [21]. The fibroblast and inflammatory cell densities were graded as following: Grade 1, <100 fibroblasts/inflammatory cells per ×400 field; Grade 2, 100–150 fibroblasts/inflammatory cells per ×400 field, Grade 3, >150 fibroblasts/inflammatory cells per ×400 field. To more stringently assess fibrotic situation, immunohistochemistry was performed with a vimentin antibody, and vimentin staining intensity was evaluated.

### 2.6. Real-time PCR

The mRNA analyses of IL-6 and TGF-β1 were performed 4 weeks after surgery in five rats randomly selected from each group. All rats were humanely euthanized, and the scar tissue from the laminectomy sites was resected. Total RNA was extracted using TRIzol reagent (Invitrogen), and the RNA (2 μg) was transcribed into cDNA by use of AMV Reverse Transcriptase. Quantitative real-time PCR (RT-PCR) was performed using the Bio-Rad MYIQ2 (Hercules, CA, USA) [22]. Based on our previous study [21], the primer sequences used in the present study were as folloing: TGF-β1 (148 bp), forward, 5′-GCCCTGCCCCTACATTTGG-3′, reverse, 5′-CTTG CGACCCACGTAGTAGACGAT-3′; IL-6 (131 bp), forward, 5′-ACCCC AACTTCCAATGCTCT-3′, reverse, 5′-TGCCGAGTAGACCTCATAGTGA CC-3′; GAPDH (169 bp), forward, 5′-TCACCACCATGGAGAAGGC-3′, reverse, 5′-GCTAAGCAGTTGGTGGTGCA-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification was performed as an internal control.

### 2.7. Macroscopic assessment of EF

Macroscopic assessment was performed 4 weeks after surgery in five rats randomly selected from each group. The surgical sites were carefully reopened, and epidural scar adhesion was evaluated in a double-blind fashion, with the results based on the Rydell classification. Grade 0, Epidural scar tissue was not adherent to the dura mater; Grade 1, Epidural scar tissue was adherent to the dura mater, but easily dissected; Grade 2, Epidural scar tissue was adherent to the dura mater, and difficultly dissected without disrupting the dura matter; Grade 3, Epidural scar tissue was firmly adherent to the dura mater, and could not be dissected [23,24].