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Potential roles of lncRNA-Cox2 and EGR1 in regulating epidural fibrosis following laminectomy

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Abstract. – OBJECTIVE: Epidural fibrosis, one of the common complications after spinal surgery, seriously affects the surgical decompression effect. Effectively inhibiting the fibrous tissue hyperplasia is pivotal to reduce the scar adhesion. Previous studies showed that early growth response 1 (EGR1) is associated with the fibroblast reactivity induced by transforming growth factor-beta (TGF-β) and plays a vital regulatory role in scar formation; however, the upstream targets and mechanisms still remain unclear. In this work, it was found that the level of long non-coding ribonucleic acid (lncRNA)-cyclooxygenase-2 (COX2) was significantly negatively correlated with EGR1 expression and the severity of the scar. Therefore, it was conjectured that lncRNA-COX2 may decrease fibroplasia and scar formation by negatively regulating EGR1.

MATERIALS AND METHODS: TGF-β was used to activate the embryonic and adult rat fibroblasts. Rats underwent laminectomy to establish the epidural fibrosis model. The changes in the levels of fibroplasia-related genes were measured and analyzed through messenger RNA (mRNA), lncRNA, and micro RNA expression profile chips. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was applied to determine the levels of EGR1 and lncRNA-COX2, and Western blotting was adopted to detect the content of EGR1, collagen I (Col-1), Col-3, and alpha-smooth muscle actin (α-SMA). The scar formation was reflected by hematoxylin and eosin (HE) staining and Masson staining, and the expression level of α-SMA in the scar tissues was measured *via* immunohistochemistry. Finally, micro-magnetic resonance imaging (MRI) was utilized to examine the different degrees of epidural fibroplasia.

RESULTS: It was found that the reactivity of embryonic rat fibroblasts to the TGF-β stimulation was different from that of adult rat fibroblasts. LncRNA-COX2 was highly expressed in the embryonic rat fibroblasts, but lowly expressed in the adult rat fibroblasts, which had negative correlations with the EGR1 level in embryonic and adult rat fibroblasts. In addition, it was revealed that the expression of EGR1 in the adult rat fibroblasts was remarkably higher than that in the embryonic rat fibroblasts after the activation with TGF-β. Meanwhile, the level of lncRNA-COX2 was lowered after the activation, especially in the adult rat fibroblasts. It was discovered in the *in-vivo* model that the degree of fibroplasia was positively associated with EGR1 level and negatively correlated with lncRNA-COX2 level.

CONCLUSIONS: The results of this research elucidated that the down-regulation of lncRNA-COX2 is involved in the epidural scar formation and related to the elevated EGR1 level which regulates the activation of fibroblasts and secretion of massive extracellular matrixes, suggesting that lncRNA-COX2 may modulate the role of fibroblasts in scar formation as an upstream action target of EGR1.

Key Words:
Epidural fibrosis, LncRNA-COX2, EGR1, Fibroblasts.

Introduction

Laminectomy is a commonly used operative method in spinal surgery, while the postoperative epidural scar adhesion severely affects the decompression effect of the operation[1]. Epidural

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fibrosis is the leading pathologic cause of postoperative scar adhesion. The release of a great number of transforming growth factor-beta (TGF-β) during repair can stimulate the fibroblasts to differentiate into myofibroblasts, which generate a large quantity of collagens and promote extracellular matrix accumulation. Of note, alpha-smooth muscle actin (α-SMA)-induced contractive scar tissues can cause nerve recompression[2].

Early growth response 1 (EGR1) is a kind of immediate response gene[3], whose DNA-binding site contains 3 zinc finger structures capable of binding to GC-rich sequences. It is controlled by hormones, neurotransmitters, growth factors, and many extracellular signals, thus participating in a series of biological processes such as cell proliferation, differentiation, and apoptosis[4,5]. Yang et al[6] have confirmed that EGR1 can accelerate the excessive synthesis of fibroblasts into collagens in the process of scar tissue repair involving TGF-β[6]. However, the specific upstream mechanism of EGR1 has not been clarified yet.

In recent years, the role of long non-coding ribonucleic acids (lncRNAs) in regulating cell functions has become a new hotspot in the studies of fibrosis and scar formation[7], while the regulation of transcription factors is a vital pathway of lncRNAs in regulating the cell functions[8,9]. As a category of functional RNAs unable to be translated into proteins, non-coding RNAs can be divided into two major types, namely, short-chain non-coding RNAs [including siRNA, micro RNA (miRNA) and piRNA] and lncRNAs, according to the size, of which lncRNAs are a category of RNAs with a transcript length of 200 nt, unable to encode proteins. LncRNAs, as important regulatory factors, can regulate gene expressions at the epigenetic, transcriptional, and post-transcriptional levels, thereby participating in multiple pathophysiological processes in organisms. Although lncRNAs have become research hotspots in the field of tumor[10,11], their mechanisms of action in scar formation are rarely reported[12]. Therefore, it was speculated that there may be a type of lncRNA associated with EGR1 expression and involved in epidural fibrous scar formation.

In this research, the reactivity of embryonic and adult rat fibroblasts to TGF-β stimulation, as well as the expressions of EGR1 and lncRNA-cyclooxygenase-2 (COX2), was analyzed. Also, the associations of varying degrees of scar hyperplasia with EGR1 and lncRNA-COX2 expressions were explored in an epidural fibrosis model.

Materials and Methods

Isolation and Culture of Primary Fibroblasts

Embryonic rat fibroblasts were extracted as follows: the rats at 10-13 days of gestation were killed by cervical dislocation, the embryo was taken out through caesarean section and put in 75% ethanol. Then, the embryo was rinsed in phosphate-buffered saline (PBS) until the liquid was clear after the surrounding capsule was eliminated. Next, the embryonic tissues were cut into pieces and added with 0.25% trypsin for digestion at 37°C for 15 min. Next, 1 mL of bovine serum was added into 10 mL of supernatant to discontinue the digestion, followed by centrifugation at a low speed for 5 min. The supernatant was discarded, added with PBS to resuspend the cells, and centrifuged again until the supernatant was clear. After filtration using a cell sieve, the erythrocytes were dissolved in 1% acetic acid solution. Then, the cells were seeded into Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) solution containing 10% fetal bovine serum (FBS) and 1% dual antibodies. As for the extraction of adult rat fibroblasts, the scar tissues from the incision on the back of the adult rats were fetched at 7 d, soaked, and disinfected in 75% ethanol. Subsequently, they were placed in DMEM containing 10% serum. 7 d later, the adherence of fibroblasts was observed, and a new culture medium was replaced for passage and continuous culture. After stimulation with TGF-β (10 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) for 24 h, the cells were used for the extraction of proteins and RNAs.

Animals and Modeling

The adult male Sprague-Dawley (SD) rats aged 6-8 weeks old and weighing about 200 g were utilized to establish the epidural fibrosis model after laminectomy. The rats were raised in cages (3 rats per cage) at 22-25°C, with humidity of 55-60% and a 12 h/12 h light/dark cycle. Also, they were given normal diets and water. Before modeling, the rats were first anesthetized by intraperitoneal injection of 10% chloral hydrate (4 ml/kg). The incision was located at the spinous process at the T10 level and the skin was cut open after skin preparation to separate the muscles. Laminectomy was performed to remove the lamina at the T10 level and the wound was flushed with normal saline after hemostasis and then sutured.

The rats subjected to the operation were raised separately until 7 d after the operation. The scar was graded according to previous reports[13]. This investigation was approved by the Animal Ethical Committee of Nanjing Medical University (NYD-2017-#467).

RNA Sequencing

The total RNAs in the embryonic and adult rat fibroblasts were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). NEBNext Ultra Directional Library Prep Kit (Illumina, San Diego, CA, USA) was used as the messenger RNA (mRNA) sequencing library. Later, the RNA sequencing was conducted on the Illumina HiSeq 1500 system. The gene expression levels were calculated using TopHat and Cufflinks software, and the enrichment analysis of the biological processes was performed by reference to the GO database for annotations with DAVID.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total RNAs were isolated from the fibroblasts and scar tissues by means of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the cDNA was synthesized by reverse transcription *via* PrimeScript Reverse Transcriptase (TaKaRa, Otsu, Shiga, China). The target gene-specific primers were utilized for qPCR, while SYBR Premix DimerEraser (TaKaRa, Otsu, Shiga, China) for amplification, from which the data were standardized using the U6 expression level. The primers applied are as follows: LncRNA-COX2: Forward: 5'-AAGGAAGCTTGGCGTTGTGA-3, Reverse: 5'-GAGAGGTGAGGAGTCTTATG-3'; EGR1: Forward: 5'-ATTGGAGGAGATGATGCT-3', Reverse: 5'-AATTAGGAAATGTTGGTGC-3'; U6: Forward: 5'-CTCGCTTCGGCAGCACA-3', Reverse: 5'-AACGCTTCACGAAT-TTGCGT-3'.

Western Blotting

Total Protein Extraction Kit (KeyGen, Nanjing, China) was adopted to extract the total proteins in the fibroblasts and scar tissues. The concentration of the samples obtained was measured using bicinchoninic acid (BCA) method (Beyotime, Shanghai, China), and the proteins were separated *via* 10% sodium dodecyl sulphate (SDS) gel electrophoresis after trimming. After that, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane at low temperature, sealed with 5% skim milk, and incubated with primary antibodies at low temperature overnight. The membrane was washed with Tris-Buffered Saline and Tween-20 (TBST) for three times the next day and reacted with secondary antibodies at room temperature for 1 h, followed by washing and enhanced chemiluminescence (ECL) assay in an exposure machine, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin as the references.

Histological and Immunohistochemical (IHC) Staining

The acquired spinal specimens were fixed in 4% paraformaldehyde, dehydrated in a gradient of ethanol, embedded in paraffin, and sliced to 5 μm-thick sections. After deparaffinization with xylene and hydration in a gradient of ethanol, the sections underwent hematoxylin and eosin (HE) staining and Masson staining in accordance with the conventional instructions. In terms of IHC staining, the sections were subjected to heat-induced epitope retrieval in citrate buffer solution at high temperature for 15 min and processing with 3% H_2O_2, followed by blocking in 5% goat serum for 1 h, and incubation with α-SMA antibody overnight. After the primary antibodies were washed away, the sections were treated using an IHC staining kit (Dako, Carpinteria, CA, USA). Finally, the color was developed *via* 3,3'-diaminobenzidine and the nuclei were counterstained by hematoxylin.

Antibodies and Concentrations

The antibodies and their concentrations applied in this experiment are as follows: for Western blotting, collagen I (Col-1; Abcam, Cambridge, MA, USA; ab34710, 1:1000), Col-3 (ProteinTech; Thermo-Fisher Scientific, Waltham, MA, USA; 22734-1-AP, 1:1000), α-SMA (Abcam, Cambridge, MA, USA; ab5694, 1:1000), EGR1 (Cell Signaling Technology; Danvers, MA, USA, 4154, 1:1000), GAPDH (ProteinTech, HRP-60004, 1:10000), β-actin (Abcam, Cambridge, MA, USA; ab8227, 1:2000) and goat anti-rabbit IgG horseradish peroxidase (HRP; YiFeiXue, YFSA02, 1:10000). For IHC staining: α-SMA (Abcam, Cambridge, MA, USA 1:100).

Micro-Magnetic Resonance Imaging (MRI)

The rats were selected randomly to receive micro-MRI examination. The rats were anesthetized by continuously inhaling halothane (3-4% for induction and 1.5-2% for maintenance) and

then fixed on the device in the prone position, with the inhalation amount of oxygen and nitrogen controlled at 0.4 L/min and 0.6 L/min, respectively. Then, a small animal MRI system (Bruker BioSpec 7T/20 USR, Ettlingen, Germany) was used for scanning and imaging. The scan parameters were set as follows: T2-weighted, 245×245 matrix, slice thickness: 1 mm, intersection gap: 1 mm, echo time: 25 ms, repetition time: 3100 ms, RARE factor: 16, flip angle: 90°C. The T2-weighted images were acquired in the axial planes by ParaVision 6.0.1 (Bruker BioSpec, Ettlingen, Germany).

Statistical Analysis

Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was made using One-way ANOVA test followed by Post-Hoc Test LSD (Least Significant Difference). All the tests were two-sided, with the significance level set at 0.05. The data were presented as mean ± SD in each group, and SPSS 18.0 (SPSS Inc. Chicago, IL, USA) software was used for all the statistical analyses.

Results

Differences in Secretions and TGF-β Stimulation Between Embryonic Rat Fibroblasts and Adult Rat Fibroblasts

Considering the different functions between fetal rat fibroblasts and adult rat fibroblasts, the fibroblasts were extracted from the embryonic rats cultured, and scar tissues of adult rats, while the proteins in the secretions of the two groups of cells were quantified before and after the TGF-β stimulation. The results indicated that there were minor differences in the levels of Col-1, Col-3, and α-SMA between the two groups of cells before the TGF-β stimulation. After TGF-β stimulation, however, it was observed that the levels of collagens and α-SMA secreted by the adult rat fibroblasts were raised markedly compared with those by the embryonic rat fibroblasts (Figure 1A and Figure 1B). Therefore, it was assumed that there may be a certain kind of gene controlling the reactivity of embryonic rat fibroblasts and adult rat fibroblasts to the TGF-β stimulation.

Differences in Multiple Gene Expressions in Embryonic and Adult Rat Fibroblasts

MiRNA, lncRNA, and mRNA sequencing was conducted to reflect the differences in the gene expressions in the embryonic rat fibroblasts and the fibroblasts in scar tissues of adult rats. According to the calculation and analysis, among the miRNAs in the two groups of cells, 18 were highly expressed, and 9 were down-regulated in the embryonic rat fibroblasts (Figure 2A). In addition, the sequencing results of lncRNA levels manifested that 17 lncRNAs were highly expressed in the embryonic rat fibroblasts. Notably, the expression of lncRNA-COX2 was

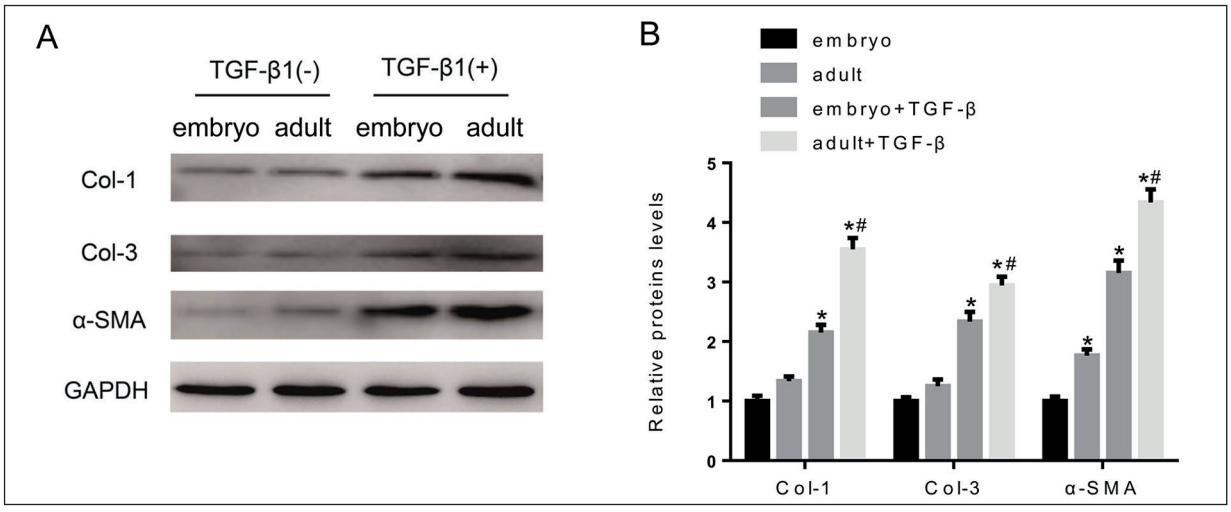

Figure 1. Differences in secretions and TGF-β stimulation between embryonic rat fibroblasts and adult rat fibroblasts. ***A,*** Protein bands of Col-1, Col-3, and α-SMA in embryonic and adult rat fibroblasts before and after TGF-β stimulation. ***B,*** Analysis of Col-1, Col-3, and α-SMA proteins. * Means statistically significant differences compared with those in the embryonic group, and # means statistically significant differences compared with those in the adult group.

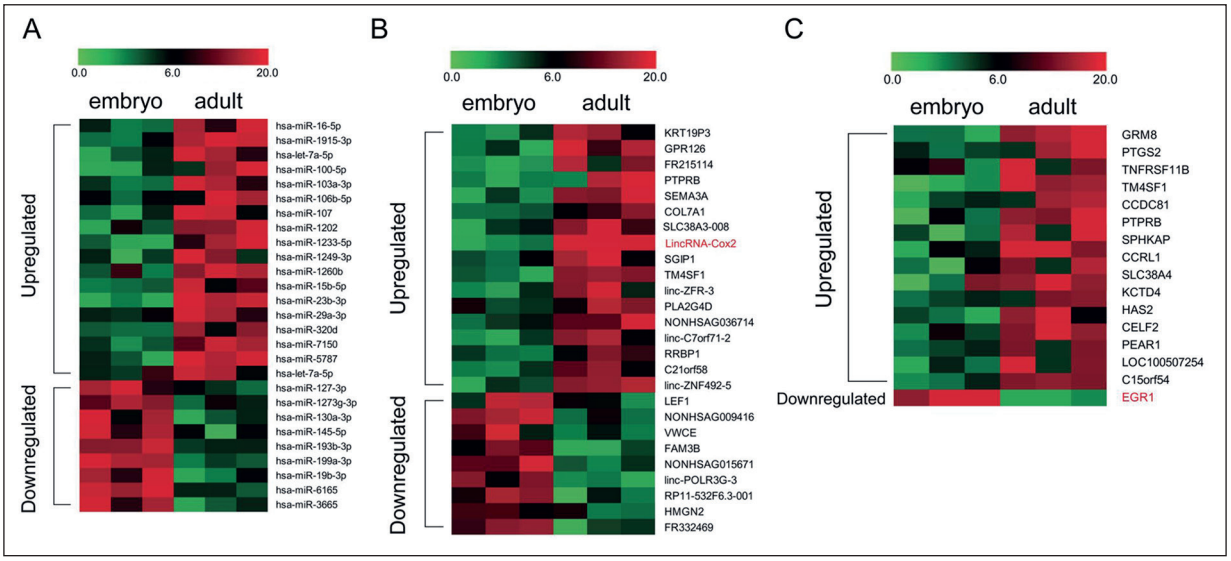

Figure 2. Differences in multiple gene expressions in embryonic and adult rat fibroblasts. ***A,*** Sequencing results of miRNA levels in embryonic and adult rat fibroblasts. ***B,*** Sequencing results of lncRNA levels in embryonic and adult rat fibroblasts. ***C,*** Sequencing results of mRNA levels in embryonic and adult rat fibroblasts.

up-regulated prominently (Figure 2B). However, the mRNA sequencing results manifested that only the EGR1 expression was evidently down-regulated in the embryonic rat fibroblasts besides 16 up-regulated mRNAs (Figure 2C). Hence, it was conjectured that the down-regulated EGR1 probably regulates the slow reactivity of the embryonic rat fibroblasts to the TGF-β stimulation, while the down-regulation of EGR1 is possibly correlated with the high expression of lncRNA-COX2.

Differential Expressions of EGR1 and LncRNA-COX2 in Embryonic and Adult Rat Fibroblasts Before and After TGF-β Stimulation

The comparisons of protein and RNA expressions of EGR1 in the embryonic and adult rat fibroblasts before and after the TGF-β stimulation revealed that the protein of EGR1 exhibited a high expression in the inactivated adult rat fibroblasts in comparison with that in the inactivated embryonic rat fibroblasts (Figure 3A). Nevertheless, the up-reg-

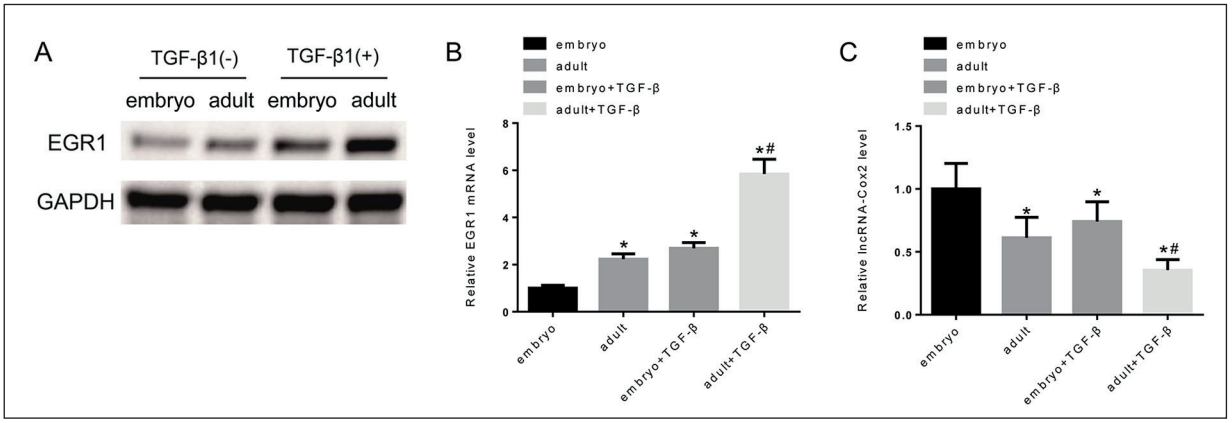

Figure 3. Differential expressions of EGR1 and lncRNA-COX2 in embryonic and adult rat fibroblasts before and after TGF-β stimulation. ***A,*** Protein bands of EGR1 in embryo and adult rat fibroblasts before and after TGF-β stimulation. ***B,*** EGR1 RNA level in embryonic and adult rat fibroblasts before and after TGF-β stimulation. ***C,*** LncRNA-COX2 RNA level in embryonic and adult rat fibroblasts before and after TGF-β stimulation. *Means statistically significant differences compared with those in the embryonic group, and # means statistically significant differences compared with those in the adult group.

ulation of EGR1 in the adult rat fibroblasts was also more significant than that in the embryonic rat fibroblasts after activation. Moreover, the RNA expression level of EGR1 was consistent with the protein expression level of EGR1, while the expressions of lncRNA-COX2 in the two groups of cells displayed the opposite trends to those of EGR1, i.e., the content of lncRNA-COX2 in the adult rat fibroblasts was notably lower than that in the embryonic rat fibroblasts before and after the TGF-β stimulation (Figure 3B and Figure 3C). The above results further verified that the sensitivity of the reactivity of embryonic and adult rat fibroblasts to TGF-β stimulation may be realized by the expression differences in lncRNA-COX2 and EGR1.

Degree of Epidural Scar Hyperplasia was Accompanied with EGR1 and LncRNA-COX2 Expressions

At 28 d after laminectomy, the rats received micro-MRI examination and the degree of scar hyperplasia was graded and grouped. Furthermore, the number of fibroblasts and collagen deposition in the scar tissues were reflected through the HE staining and Masson trichrome staining, the content and distribution of α-SMA was presented by the IHC staining. It was shown that the area of mild epidural scar tissues was relatively small, with a few fibroblasts and collagen fibers, as well as apparently low α-SMA expression. In moderate hypertrophic scar, a larger area of hypertrophic scar tissues, significantly greater numbers of fibroblasts and remarkably higher collagen deposition and α-SMA level than those in the Mild group were observed. However, there was a large area of scar tissues that compressed the spinal cord tissues in Severe group. A great number of fibroblasts, compact collagen structure, and high expression of α-SMA could also be noticed in the sections in Severe group (Figure 4A). Besides, in terms of the tissue proteins, it was found that the protein expressions of Col-1, Col-3, and EGR1 were raised in Mild, Moderate, and Severe groups in sequence (Figure 4B). Of note, it was discovered in the RNA sequencing results in three groups of tissues that the gradually increased transcription level of EGR1 was accompanied with the gradually decreased RNA expression of lncRNA-COX2 among the three groups (Figure 4C). Therefore, the elevated expression level of EGR1 aggravates the epidural scar hyperplasia, and its elevation may be closely related to the down-regulation of lncRNA-COX2.

Discussion

We showed that epidural fibrosis seriously influences the satisfaction of decompressive nerve operation in spinal surgery. This leads to the failure of surgical decompression and even increases the probability of the second operation, which is of great significance in preventing the postoperative scar adhesion. The epidural scar formation after laminectomy is primarily caused by abnormal proliferation, hyperactivation, and massive collagen deposition in local fibroblasts[14,15]. Therefore, how to inhibit the abnormal proliferation and hyperactivation of fibroblasts becomes the key point. Currently, the major treatment means include the topical application of biomaterials to obstruct the infiltration and aggregation of fibroblasts[16], topical application of medicines repressing fibroblast proliferation[17], local application of anti-tumor drugs promoting fibroblast apoptosis[18-20], wrapping of the nerve root and dura mater in the operative region using fluid or semi-fluid substances[21], as well as immunotherapy[22]. However, none of them possesses a completely preferable efficacy. According to the previous studies[23] over the past few years, mitomycin C (MMC-C) and tacrolimus (FK506) can effectively suppress the fibroblast proliferation and collagen synthesis, and accelerate fibroblast apoptosis, thus evidently inhibiting the epidural scar formation[23]. Nevertheless, the topical application of MMC-C and FK506 produces toxic effects on peripheral nerve tissues, and the excessive fibroblast apoptosis delays the wound healing and stimulates the deposition of collagen and cell-matrix to expand the scar. All these factors severely restrict the clinical application of those medicines. Hence, it is urgent to discover a specifically recognized molecular mechanism and explore drug targets to ameliorate the epidural scar adhesion. TGF-β is not only a pivotal player in tissue repair after trauma or operation but also a crucial factor for scar formation. The sensitivity of fibroblasts to the TGF-β stimulation decides the degrees of collagen deposition and scar contracture, thus leading to regeneration disorder at the wound. It has been reported[24] that the scarless repair of fetal skin can be realized because the fetal skin tissues have a weaker response to the TGF-β stimulation compared to adult skin during the wound repair. Therefore, it was presumed that such a difference may be attributed to the changes in gene expressions in fetal and adult fibroblasts. EGR1 serves as a

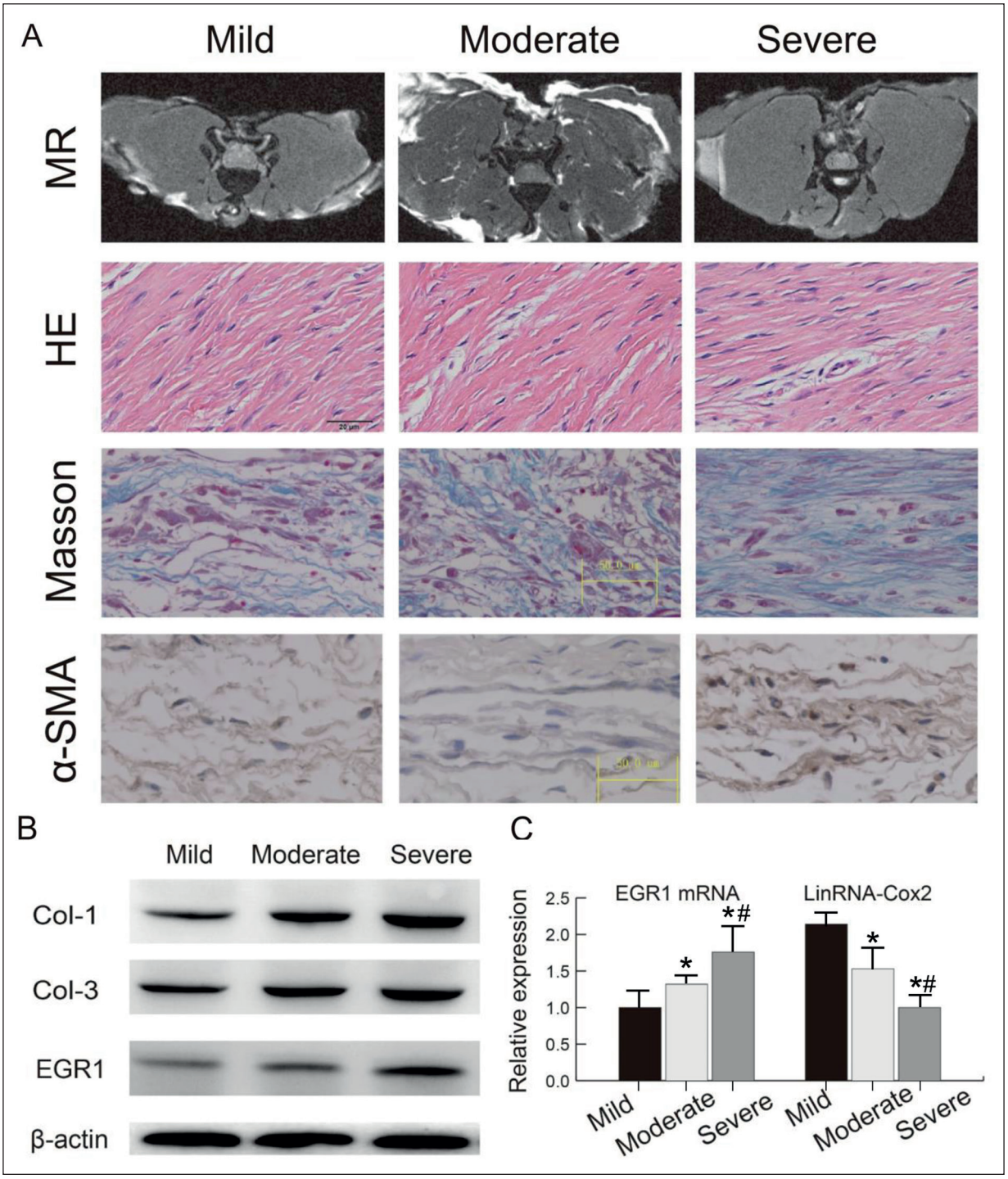

Figure 4. Degree of epidural scar hyperplasia is accompanied with EGR1 and lncRNA-COX2 expressions. ***A,*** Micro-MRI, HE, Masson, and IHC staining of α-SMA in Mild, Moderate, and Severe groups (magnification: 200X). ***B,*** Protein bands of Col-1, Col-3, and EGR1 in Mild, Moderate, and Severe groups. ***C,*** RNA levels of EGR1 and lncRNA-COX2 in Mild, Moderate, and Severe groups. * Means statistically significant differences compared with those in Mild group and # means statistically significant differences compared with those in Moderate group.

coupling molecule between transient stimulation of extracellular environments and long-term response to cells. A variety of bio-stimulations can induce the rapid expression of EGR1 gene to participate in the cellular activities. Although EGR1 mediates the epidural fibrosis, the targets

controlling EGR1 during the process still remain unknown. As an intergenic lncRNA with a length of 2.15 kb, lncRNA-COX2 is located at Chr1 186647401-186649559 in human genome[25]. LncRNA-COX2 can be induced by pattern recognition receptors in innate immune responses, thereby participating in various immuno-inflammatory responses[26]. However, whether lncRNA-COX2 has any relation to scar formation has not been reported yet.

In this work, the genes were compared between the two groups of fibroblasts in different developmental stages. It was found that lncRNA-COX2 and EGR1 had expression differences. The changes in collagen secretion, lncRNA-COX2, and EGR1 in the embryonic and adult rat fibroblasts before and after the TGF-β stimulation were further testified. Results demonstrated that the changes in EGR1 modulated the secretion and reactivity to the TGF-β stimulation of the embryonic and adult rat fibroblasts. Besides, the changes in EGR1 were correlated with the expression changes in lncRNA-COX2. The correlation analysis on lncRNA-COX2 and EGR1 levels in the epidural scar tissues after laminectomy indicated that lncRNA-COX2 had a negative correlation with the severity of the scar and was prominently negatively correlated with EGR1 level. On the basis of these findings, it was reasonable to presume that lncRNA-COX2 plays a negative regulatory role in scar formation; however, whether lncRNA-COX2 has a potential regulatory effect on EGR1 expression and its regulatory mode are still unclear and they need to be further explored and demonstrated.

Conclusions

The changes in lncRNA-Cox2 and EGR1 expressions in embryonic and adult rat fibroblasts and the TGF-β stimulation to fibroblasts induced EGR1 elevation and lncRNA-COX2 reduction. The severity of epidural scar was associated with increasing EGR1 and decreasing lncRNA-COX2. LncRNA-Cox2 may have a potential modulating effect on EGR1 to ameliorate the epidural fibrosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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