Analysis of pathogenesis of the thickened ligamentum flavum in lumbar spinal canal stenosis

博士論文

Analysis of pathogenesis of the thickened ligamentum flavum in lumbar spinal canal stenosis

（腰部脊柱管狭窄症における肥厚した黄色靭帯の病態解析）

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1. Abstract

Lumbar spinal canal stenosis (LSCS) is one of the most common spinal disorders in elderly patients. Thickening of the ligamentum flavum has been considered a major contributor to the development of LSCS. Although previous studies have reported some growth factors, cytokines, matrix metalloproteinases (MMPs), and tissue inhibitors of matrix metalloproteinases (TIMPs) may play important roles in the pathogenesis of the thickened ligamentum flavum, its etiology is still unclear. The aim of this study was to analyze changes in the thickened ligamentum flavum and clarify their etiology.

The ligamentum flavum samples were collected from 20 patients with LSCS (LSCS group) and 10 patients with lumbar disc herniation (LDH group) as a control. The thickness of the ligamentum flavum was measured histologically. The amount of elastic fibers and proteoglycans were assessed by Elastica-Masson staining and alcian blue staining, respectively. Gene and protein expressions related to elastogenesis, fibrosis, inflammation, chondrogenesis, and proteoglycan synthesis were analyzed by quantitative reverse transcription polymerase chain reaction and immunohistochemistry. The total genes of the two groups were compared by DNA microarray analysis.

The thickness of the ligamentum flavum was significantly greater in the LSCS group compared with that in the LDH group. The amount of elastic fibers was smaller in the LSCS group and these changes were prominent on the dorsal side of the ligamentum flavum.

Staining intensity of alcian blue was significantly stronger in the LSCS group. The gene expressions related to elastogenesis were significantly lower in the LSCS group. The gene and expressions related to fibrosis were significantly higher in the LSCS group, however, the immunoreactivities of collagen types I and III were weaker on the dorsal side of the ligamentum flavum in the LSCS group. The gene and protein expressions related to chondrogenesis and proteoglycan synthesis were significantly higher in the LSCS group. There was no significant difference in the gene expressions related to inflammation between the two groups.

Elastic fiber formation decreases in the ligamentum flavum of the patients with LSCS, which may lead to loss of elasticity. Synthesis of collagenous fibers and degradation of the elastic and collagenous fibers are both accelerated in the ligamentum flavum of patients with LSCS, which may be the reason for hypertrophy of the tissue. In addition, chondrogenesis and proteoglycan synthesis may have critical roles in the pathogenesis of the ligamentum flavum thickening.

2. Introduction

Lumbar spinal canal stenosis (LSCS) is one of the most common spinal disorders in elderly patients. The concept of LSCS was made by Verbiest in 1954. He reported that narrowing of the bony vertebral canal caused neurological symptoms.[1] It is estimated that 2.4 million people have this disease in Japan. LSCS causes low back pain, leg pain and numbness, intermittent claudication, and urinary dysfunction by compression of the cauda equina and the nerve roots due to narrowing of the spinal canal, which leads to severe disability in the activities of daily living. Conservative treatments such as exercise, medication, and nerve block are performed and operation is needed in severe cases.[2,3] LSCS occurs as a result of degenerative changes of the lumbar spine, including buldging of the intervertebral discs, bony proliferation of the facet joints, and thickening of the ligamentum flavum.[4,5] The ligamentum flavum is a yellow elastic ligament which covers the posterior and lateral walls of the spinal canal and thickening of the ligamentum flavum has been considered as a major contributor to the development of LSCS.[6,7] Altinkaya used the words “thickness” and “hypertrophy” differently. Thickness may increase by buckling without a change in the mass of the ligamentum flavum. The buckling of the ligamentum flavum into the spinal canal can occur after disc collapse.[8] Although thickening of the ligamentum flavum is due to tissue hypertrophy or buckling, histologic changes leading to tissue hypertrophy have been reported, there remains a controversy.[7-9] Previous studies have indicated that thickened ligamentum

flavum shows a loss of elastic fibers and an increase of collagen fibers.[4,9-11] Therefore, fibrosis has been thought to be a main etiology of thickening of the ligamentum flavum and some factors related to fibrosis have been reported. Nakatani et al. reported that mechanical stretching force promoted TGF-β1 production by ligamentum flavum cells which might lead to fibrosis of the ligament.[4] Zhong et al. reported that connective tissue growth factor (CTGF) expression was higher in the thickened ligamentum flavum.[11] Zhang et al. reported that higher platelet-derived growth factor-BB (PDGF-BB) existed in the thickened ligamentum flavum.[12] Park et al. reported that increased matrix metalloproteinases (MMPs) might be related to the elastin degradation and fibrosis of the ligamentum flavum.[13] Park et al. also reported that increased tissue inhibitors of matrix metalloproteinases (TIMPs), especially TIMP-2, might cause fibrosis of the ligamentum flavum by inhibiting MMP activity.[10] The reports of thickened ligamentum flavum are small in number and only a few except fibrosis. Yoshida et al. reported that Type II collagen was identified in the thickened ligamentum flavum and chondrometaplasia might be one cause of thickening.[6] Sairyo et al. reported that inflammatory cytokines were confirmed to be expressed in the ligamentum flavum and repetitive inflammation might cause scar accumulation, which would gradually lead to an increase in thickness.[14] Though some growth factors, cytokines, MMPs, and TIMPs may play important roles in the pathogenesis of thickening of the ligamentum flavum, its mechanism is still unclear. Further, elastic fibers and proteoglycans have important roles in some pathological

conditions.[15,16] Decrease of elastic fibers leads to a loss of the elasticity of tissues. It has been reported that elastic fibers are reduced and elastic system components are disrupted in normal and hypertrophic scars, which is similar to the conditions of the thickened ligamentum flavum.[17,18] Some proteoglycans increase in fibrotic diseases including scars.[19] Recent studies have indicated that accumulation of some glycosaminoglycans and proteoglycans relates to impaired elastic fiber formation.[20-22] However, the changes of elastic fibers and proteoglycans in the thickened ligamentum flavum are not known.

3. Purpose

The aim of this study was to elucidate the pathogenesis of the thickened ligamentum flavum using immunohistochemistry, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and DNA microarray analysis. It will lead to development of prevention or new drug therapies for LSCS.

4. Materials and Methods

4.1. Samples

The protocols of this study were approved by institutional review boards of Takeda General Hospital (approval number; 2011-05). Twenty patients with LSCS necessitating decompressive surgery due to neurogenic claudication with thickening of the ligamentum flavum on magnetic resonance imaging (MRI) comprised the study group (LSCS group). The control group consisted of 10 patients with lumbar disc herniation (LDH) necessitating surgery without thickening of the ligamentum flavum on MRI (LDH group). The average age was 72.9 years (range 57-84, 10 males and 10 females) in the LSCS group and 64.6 years (range 47-77, 4 males and 6 females) in the LDH group. The difference of age distribution between the two groups was marginal but not statistically significant (P=0.0542). The thickness of the ligamentum flavum was evaluated using MRI (MAGNETOM Verio, 3.0T-unit; Siemens, Erlangen, Germany, or Signa HDxt, 1.5T-unit, GE Healthcare, Milwaukee, WI) in supine position. On the T1-weighted axial images through the facet joint, the ligamentum flavum was clearly seen as a low-signal intensity mass just ventral side of the facet joint. A vertical line was drawn to the anterior side of the lamina to measure the thickness of the ligamentum flavum. The thickest portion of the ligamentum flavum was measured as thickness (Figure 1).

4.2. Tissue preparation

Fenestration was performed and the ligamentum flavum was obtained en-bloc during surgery. It was sagittally cut and evaluated histologically. The tissues were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.4 containing 18% sucrose for cryoprotection. The frozen tissue was cut into 5-μm sections and was stained by following methods. The Elastica-Masson stained sections were used to evaluate the thickness of the ligamentum flavum and the amount of elastic fibers. The thickest portion of the ligamentum flavum was measured as a representative thickness. Regions of interest (ROI) in an area of 0.25 mm^2 were selected from 10 sites in each ligamentum flavum: 5 on the dural side and 5 on the dorsal side as previously described.[9] ROIs on each side were randomly selected (Figure 2). The area in dark purple color within the ROI, which corresponds to the ratio of elastic fibers, was calculated with use of ImageJ 1.47 software (National Institutes of Health, Besthesda, MD, USA). The amount of proteoglycans was assessed by alcian blue staining intensity. The changes of the elastic fibers were evaluated by high power images of Elastica-Masson staining and compositional changes of elastic fibers and proteoglycans were compared using these staining.

4.3. Immunohistochemistry (IHC)

The sections were dried and post-fixed with 4% PFA for 10 minutes at room temperature, and immersed in 3.0% hydrogen peroxide for 10 minutes. The slides were incubated with 0.1% Trypsin 0.1% $CaCl_2$/Tris Buffer at room temperature for 10 minutes.

Endogenous immunoglobulins were blocked by incubation with 10% normal goat serum (Nichirei, Tokyo, Japan) in PBS. The slides were incubated with antibodies (Table 1). The final detection step was carried out using 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St Louis, USA), 0.1 M imidazole, and 0.03% hydrogen peroxide. These slides were counterstained with hematoxylin. For negative controls, normal mouse, goat, or rabbit IgG was used as a primary antibody.

4.4. RNA extraction and purification

All the samples cut into small pieces were immediately placed in a vessel containing 3-ml QIAzol (Qiagen, Hilden, Germany). They were stored immediately in a liquid nitrogen tank until RNA extraction. The frozen samples were homogenized with a Polytron® (Kinematica AD, Switzerland). The total RNA of the homogenate was purified using RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacture's instruction.

4.5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Complementary DNA was synthesized using Cloned avian myeloblastis virus (AMV) first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacture's instruction. Gene expressions were evaluated quantitatively by real-time polymerase chain reaction (PCR) on a LightCycler (Roche Diagnostics, Basel, Switzerland). SYBR green was used for quantification of the amplified DNA. The cycling conditions were: 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 59°C for 30 sec and 72°C for 1 min.

At the end of the run, the melting temperature of the amplified product was measured to confirm its homogeneity. PCR efficiencies and relative expression levels of genes related to elastogenesis, fibrosis, inflammation, chondrogenesis, and proteoglycan synthesis as a function of elongation factor 1α1 (EF1α1) expression were calculated.[23] The primer sequences for expression analyses are in Table 2, 3, and 4.

4.6. DNA microarray analysis

Eight LSCS samples and three LDH samples were used for DNA microarray analysis. After treatment with DNase I (Invitrogen), RNAs were amplified using Amino Ally MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX) and then labeled with Cy3 and Cy5. Whole Human Genome Microarray Kit 4 × 44 K (Agilent, Santa Clara, CA) was applied to the Cy3- and Cy5-labeled amplified RNAs, which were then competitively hybridized at 65°C for 17 hours.[24] The microarray was scanned with GenePix 4000B (Molecular Devices, Downingtown, PA). The scanned image was analyzed with GenePix Pro 6 software (Molecular Devices).

4.7. Statistical analyses

Differences between the LSCS and LDH groups were compared by unpaired t-test (thickness) and by Mann–Whitney's U test (age, qRT-PCR). Data were expressed as mean ± standard deviation (SD). Tests were two-sided and a value of $P < 0.05$ was accepted as statistically significant.

5. Results

5.1. Thickness of the ligamentum flavum

On MRI, the mean thickness of the ligamentum flavum from the LSCS group (5.12 ± 0.9 mm, range: 3.93-7.45) was significantly greater than that of the LDH group (2.93 ± 0.59 mm, range: 1.69-3.73) ($P < 0.001$).

Histologically, the mean thickness of the ligamentum flavum from the LSCS group (4.77 ± 0.79 mm, range: 3.91-7.16) was significantly greater than that from the LDH group (3.17 ± 0.50 mm, range: 2.31-3.77) ($P < 0.001$).

5.2. Elastic fibers in the ligamentum flavum

The amount of elastic fibers was significantly smaller in the LSCS group, and these changes were prominent on the dorsal side of the ligamentum flavum. (Table 5, Figure 3A-D). Elastic fibers were thick and well regulated in the LDH group. However, they were sparse, thin, and fragmented in the LSCS group (Figure 4A and B). The gene expressions of fibrillin-2 and DANCE (fibulin-5) were significantly lower in the LSCS group compared with those in the LDH group. There was no statistical difference in the gene expressions of elastin, fibrillin-1, EMILIN-1, and fibulin-1 between the two groups (Table 6).

5.3. Fibrosis and inflammation in the ligamentum flavum

The gene expressions of COL1A2, COL3A1, CTGF, and Cysteine-rich angiogenic inducer 61 (CYR61) were significantly higher in the LSCS group. There was no statistical

difference in the gene expressions of COL1A1, TGF-β1, PDGF-BB, α-smooth muscle actin (α-SMA), tumor necrosis factor (TNF)-α, and interleukin (IL)1-β between the two groups (Table 7). The gene expressions of MMP-2 and TIMP-2 were significantly higher in the LSCS group. There were no statistical differences in the gene expressions of MMP-1, 3, 9, 13, and 14, TIMP-1 and 3 between the two groups (Table 8). There was no apparent difference in immunoreactivities of collagen types I and III on the dural side between the two groups. However, the immunoreactivities were weaker on the dorsal side in the LSCS group (Figure 5A-F). Immunoreactivities of CTGF and MMP-2 were stronger in the LSCS group (Figure 6A-D).

5.4. Chondrogenesis and proteoglycan synthesis in the ligamentum flavum

The intensity of alcian blue staining was significantly stronger in the LSCS group. These changes were more evident on the dorsal side of the ligamentum flavum in accordance with the decreased elastic fibers (Figure 7A and B). The gene expressions of COL2A1, decorin, lumican, versican, and osteoglycin (OGN) were significantly higher in the LSCS group. There were no statistical differences in the gene expressions of COL10A1, SOX9, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4 and 5, aggrecan, biglycan, and fibromodulin between the two groups (Table 9). Immunoreactivities of collagen type II, decorin, lumican, versican, and OGN were stronger in the LSCS group (Figure 8A and B, 9A-D, 10A-D). There was no apparent difference in immunoreactivities of aggrecan,

biglycan, and fibromodulin between the two groups (Figure 11A-F).

5.5. Gene expression profiles

To characterize microarray expression profiles, probes whose expressions changed by more than twofold up-regulation in the majority of the ligamentum flavum in the LSCS group or less than a half down-regulation in all the ligamentum flavum in the LSCS group compared with those in the ligamentum flavum in the LDH group were selected.[25] The gene

expressions of 12 genes were significantly higher (Table 10) and those of 22 genes were significantly lower (Table 11) in the LSCS group, among approximately 20,000 genes. Notably, fibrotic factors such as COL5A2 and COL1A2, chondrogenic factors such as COL11A1 and COL2A1 were significantly higher in the LSCS group.

6. Discussion

The ligamentum flavum covers the posterior and lateral walls of the spinal canal. As the ligamentum flavum becomes thickened, it will compress the nerve roots and the cauda equina.[7] Removal of the thickened ligamentum flavum is a main target of surgical treatment of LSCS.[12] Some authors have evaluated the thickness of the ligamentum flavum using MRI or computed tomography (CT) and indicated that the ligamentum flavum is significantly thicker in LSCS.[10-12,26-28] This study included the thickened ligamentum flavum on MRI (LSCS group) and non-thickened ligamentum flavum on MRI (LDH group). There have been no definition of thickening of the ligamentum flavum and there remained a possibility that the mild thickened ligamentum flavum was included in the LDH group. However, the results of the thickness of the ligamentum flavum in this study were almost same with the previous reports.[10-12,26-28] Some authors have argued that the ligamentum flavum thickening is due to tissue hypertrophy, but others to the buckling of the ligamentum flavum.[5,8,11,26] To confirm hypertrophy of the ligamentum flavum, we assessed the thickness of the ligamentum flavum histologically and it was significantly greater in the LSCS group. This result indicated that the ligamentum flavum was thickened in LSCS, which was due in part to tissue hypertrophy. MRI or CT images of the patients in this study can detect tissue hypertrophy together with buckling of the ligamentum flavum in the LSCS group.

Elastic fibers are major components of the extracellular matrix in elastic connective

tissues such as large arteries, skin, lungs, and ligaments. They give these tissues resilience and elasticity to withstand repeated cycles of stretch and recoil through life.[29] The two major structural components of elastic fibers are fibrillin-rich microfibrils and elastin, and other proteins control the process of their formation.[17,20] Elastic fibers are reduced in pathological conditions, such as aortic aneurysms, pulmonary emphysema, and scars.[15,18] Accelerated degradation or reduced synthesis of elastic fibers results in a loss of the elasticity of tissues.[30] The normal ligamentum flavum is a well-defined elastic structure with 80% elastic fibers and 20% collagen fibers.[31] Upon thickening, the ligamentum flavum shows a loss of elastic fibers and an increase of collagen fibers, resulting in fibrosis.[11] Elastic fibers are made up of a central core of amorphous hydrophobic cross-linked elastin surrounded by fibrillin-rich microfibrils. Fibrillin is thought to act as a scaffold for the deposition of tropoelastin (the soluble precursor of mature elastin) onto microfibrils. DANCE is also required for elastic fiber assembly.[15,17,20] Decreased expressions of fibrillin-2 and DANCE indicated disrupted elastogenesis in the thickened ligamentum flavum in this study. Sparse, thin, and fragmented elastic fibers might signify a disturbance in maturation in the thickened ligamentum flavum. Kosaka et al. divided the ligamentum flavum into 2 layers (dural and dorsal sides) and indicated that elastic fibers decreased mainly on the dorsal side.[9] Sairyo et al. reported that the dorsal layer of the ligamentum flavum was always subjected to more load than the dural layer during lumbar motion. [7] They speculated that the ligamentum flavum thickening might be a

mechanically induced condition.

The gene expressions of collagen types I and III were significantly higher in the LSCS group by qRT-PCR. These results indicated fibrotic process was prominent in the thickened ligamentum flavum. Some factors related to fibrosis, such as TGF-β1, CTGF, and PDGF-BB, have been reported to be involved in the thickened ligamentum flavum.[11,12,26] The gene expression of CTGF was significantly higher in the LSCS group. However, there was no statistical difference in the gene expressions of TGF-β1 and PDGF-BB between the two groups in this study. Sairyo et al. reported the gene expression of TGF-β1 decreased as the ligamentum flavum thickness increased. TGF-β1 has been expressed only during the early phase of the scarring process and it might contribute to thickening of the ligamentum flavum during the early stages.[7] LSCS is a disorder with a long duration and the expression of TGF-β1 may depend on stages of LSCS. Zhang et al. divided the ligamentum flavum into 2 layers (dural and dorsal layers) and reported higher gene expression of PDGF-BB in the dorsal layers.[12] This result indicated that fibrogenic process might occur mainly on the dorsal side of the ligamentum flavum. There was no statistical difference of the gene expression of PDGF-BB between the two groups in this study. The layers of the LF were not divided in this study, which might make the difference smaller between the two groups. Regarding the angiogenic factors, CYR61 is highly expressed in granulation tissues during cutaneous wound healing.[32] CYR61 drives fibroblasts into senescence and up-regulates the expression of

antifibrotic genes to restrict fibrosis during tissue repair.[33] The gene expression of CYR61 was significantly higher in the LSCS group. This data supported an existence of fibrosis in the thickened ligamentum flavum. Inflammation has been considered to be the initial event which causes scar formation in the ligamentum flavum.[14] However, there was no difference in the gene expressions of TNF-α and IL1-β between the two groups in this study. These results might depend on disease stages.

MMPs are a family of at least 24 peptidases that are collectively responsible for the degradation of extracellular matrix during tissue remodeling.[34,35] TIMPs are the major endogenous regulators of MMPs activities in tissues, and four homologous TIMPs (TIMPs-1 to 4) have been identified.[36] Some MMPs and TIMPs, such as MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, and TIMP-2 were reported to be increased in the thickened ligamentum flavum.[10,13,37] The gene expressions of MMP-2 and TIMP-2 were significantly higher in the LSCS group, whereas the others were not significantly different between the two groups. Disease stages, differences of age distribution may have affected the results. MMP-2, which has an ability to degrade elastin, has been reported to be increased in human abdominal aortic aneurysm.[38] The amount of elastic fibers in the LSCS group was smaller than in the LDH group. In addition, these changes were prominent on the dorsal side of the ligamentum flavum. Up-regulated MMP-2 might be associated with a loss of elastic fibers in the thickened ligamentum flavum. MMP-2 is well-known as a gelatinase and digests denatured collagens.

Some authors have reported that MMP-2 also has an ability to digest interstitial collagens.[35,39] Though the gene expressions of collagen types I and III were significantly higher in the LSCS group, the immunoreactivities of collagen types I and III were weaker on the dorsal side of the ligamentum flavum in the LSCS group. These contradictory results were explained by the ability of MMP-2 to digest collagens. Up-regulated MMP-2 might degrade increased collagens and TIMP-2 is the major inhibitor of MMP-2.[40] Collagen synthesis and degradation might be accelerated in the thickened ligamentum flavum, which might lead to scar formation.

Proteoglycans consist of a core protein to which at least one glycosaminoglycan chain is attached. They maintain the structure of the extracellular matrix and play important roles in the physiology and biomechanical function of tissues.[16,41] Alcian blue staining intensity was stronger in the LSCS group. This result supported increased proteoglycans in the thickened ligamentum flavum as previously reported.[42] And the gene expression of collagen type II was significantly higher in the LSCS group. These results indicated chondrogenic process in the thickened ligamentum flavum. These changes have been also reported to occur at the insertion site of the other ligaments and considered as a mechanical response.[6,9] There was no significant difference in the gene expressions of SOX9, aggrecan, and COL10A1, which were specific in the articular cartilage, between the two groups. These results indicated that the chondrogenic gene expression patterns in the thickened ligamentum

flavum were different from those in the articular cartilage. Proteoglycans are divided into two main groups, small leucine-rich proteoglycans (SLRPs) and large proteoglycans by the size of their core proteins. Decorin, lumican, and osteoglycin are members of SLRPs and versican belongs to large proteoglycans.[16] They are detected in cartilage matrix during chondrogenesis.[43,44] The gene expressions of decorin, lumican, osteoglycin, and versican were significantly higher in the LSCS group. These results might support chondrogenic process in the thickened ligamentum flavum. There remains other possibility in pathogenesis of increased proteoglycans. Increased proteoglycans have been reported to be associated with pathological processes, such as wound healing, organ fibrosis, and tumor development.[16,41,45-47] An increased decorin expression has been shown in tissues of some fibrotic diseases, such as postmyocardial infarction, liver fibrosis, and renal fibrosis.[48-50] Lumican has been reported to increase in cardiac fibrosis and tumors.[46,51] Versican has been reported to increase in many diseases, such as inflammatory lung disorders, hypertrophic scars, and tumors.[19,52,53] Increased versican has also been detected in injured ligaments.[54] Though there have been few reports of increased osteoglycin, it has been reported that osteoglycin regulates cellular growth and an increased expression deceases the metastatic capability of mouse hepatocarcinoma.[55,56] Proteoglycans, such as decorin, lumican, osteoglycin, and versican increased in the LSCS group in this study. And these changes were prominent on the dorsal side of the ligamentum flavum in accordance with the decreased

elastic fibers. These proteoglycans increase in the repair process or fibrotic conditions as described above. Our results supported the theory that thickening of the ligamentum flavum is a result of micro tissue injuries due to lumbar motion.[7] Mechanical stress during lumbar motion might cause repetitive injury and subsequent accelerated healing process might increase proteoglycans in the ligamentum flavum. Chondrogenic process and proteoglycan synthesis might be one of the main etiologies of the thickened ligamentum flavum.

Considering increased proteoglycans, there are other possibilities in the pathogenesis of the ligamentum flavum thickening. Decorin inhibits TGF-β1, which plays a central role in fibrosis, and TGF-β1 upregulates decorin production.[45,57] Decorin is reduced in repairing ligaments and postburn hypertrophic scars, leading to TGF-β1 activation and fibrosis.[54,58] However, the expression of decorin during wound repair is very complex. Sayani et al. reported that the expression of decorin in burn wounds is low up to a year and increases from 1 to 3 years after the injury.[59,60] An increased decorin expression has been shown in tissues of some fibrotic diseases.[48-50] These results mean that decorin has a protective role in organ fibrogenesis by inhibiting TGF-β1 activity.[45] Sairyo et al. reported that the expression of TGF-β and ligamentum flavum thickness show a negative linear relationship.[7] LSCS is a disease with a long duration and an increased decorin may inhibit TGF-β activity in the thickened ligamentum flavum. Ikeda et al. reported that elastic fiber assembly is disrupted by excessive accumulation of chondroitin sulphate glycosaminoglycans in keloid lesions.[20]

Hinek et al. reported that Hurler disease causes accumulation of dermatan sulfate glycosaminoglycans, which impairs elastogenesis by inhibition of elastic fiber assembly.[22] Chondroitin sulfate and dermatan sulfate are chains of decorin and versican.[16] Chronic obstructive pulmonary disease is characterized by a loss of elastic fibers in the alveolar wall and these changes are considered to be related to increased versican.[21] Increased proteoglycans might inhibit elastic fiber assembly, which accelerated the loss of elastic fibers, and increase the thickness of the ligamentum flavum. Decreased elasticity may cause buckling of the ligamentum flavum.

From DNA microarray analysis, gene expressions related to fibrosis, such as COL5A2 and COL1A2, chondrogenesis, such as COL11A1 and COL2A1, were significantly higher in the LSCS group. Considering other genes in the microarray analysis, there are some possibilities in the pathogenesis of the ligamentum flavum thickening. The gene expression of OGN was significantly higher in the LSCS group alike to the result of qRT-PCR. The WNT1 inducible signaling pathway protein 1 (WISP1) and fibronectin (FN) are expressed in fibrotic disorders.[61,62] The gene expressions of WISP1 and FN were significantly higher in the LSCS group. These data might support fibrogenic process in the thickened ligamentum flavum. Dipeptidyl peptidase-4 (DPP4) is reported to degrade denatured collagen.[63] The gene expression of DPP4 was significantly higher in the LSCS group. This data might support collagen degradation process in the thickened ligamentum flavum.

Limitations of this study are as follows: (1) the samples were contracted while immersed in PFA for tissue preparations, (2) the average age of the LSCS group was higher than the LDH group and we cannot exclude the possibility that the natural aging process affected the results, (3) LSCS is a disease with a long duration. Samples might be obtained from several stages of the disease and affect the results, (4) protein expressions are not evaluated quantitatively.

My hypothesis of the pathogenesis of the thickened ligamentum flavum obtained from this study is as follows: (1) mechanical stress during lumbar motion causes repetitive injury of ligamentum flavum, which leads to degradation of elastic fibers and coexistence of synthesis and degradation of collagen fibers, (2) accelerated healing process causes accumulation of proteoglycans which leads to chondrometaplasia, (3) decreased elastogenesis and disrupted elastic fiber assembly caused by increased proteoglycans leads to loss of elastic fibers, (4) hypertrophy of the tissue due to accumulation of the extracellular matrix in this process and buckling of the tissue due to loss of elasticity causes thickening of the ligamentum flavum (Figure 12).

7. Conclusion

In conclusion, the present study suggested as follows: (1) The ligamentum flavum was thickened in LSCS, which was due in part to tissue hypertrophy, (2) Elastic fibers decreased in the thickened ligamentum flavum. Increased MMP-2 might be associated with a loss of elastic fibers. Further, Fibrillin-2 and DANCE decreased in the thickened ligamentum flavum, which indicated decreased elastogenesis, (3) The gene expressions of COL1A2, COL3A1, CTGF, and CYR61 increased in the thickened ligamentum flavum, which indicated fibrosis, (4) Immunoreactivities of collagen type I and III were weaker on the dorsal side of the thickened ligamentum flavum, which indicated degradation of collagen fibers. Increased MMP-2 might degrade increased collagen fibers, (5) TIMP-2 also increased in the thickened ligamentum flavum, which might inhibit MMP-2. Collagen synthesis and degradation might be accelerated in the thickened ligamentum flavum like scar formation, (6) Proteoglycans, such as decorin, lumican, versican, and osteoglycin increased in the thickened ligamentum flavum. These proteoglycans might increase in the process of accelerated collagen synthesis and degradation, (7) Collagen type II increased in the thickened ligamentum flavum, which indicated chondrometaplasia along with increased proteoglycans, (8) Increased proteoglycans might inhibit elastic fiber assembly and accelerate the loss of elastic fibers. Decreased elasticity may cause buckling of the ligamentum flavum, (9) DNA microarray analysis was performed. The expressions of 12 genes increased in the thickened ligamentum flavum among

approximately 20,000 genes. Notably, fibrotic factors such as COL5A2 and COL1A2, chondrogenic factors such as COL11A1 and COL2A1, and OGN increased in the thickened ligamentum flavum.

Thickening of the ligamentum flavum is due to hypertrophy and a loss of elasticity of the tissue. Accumulated proteoglycans are thought to be associated with these changes. Decomposing proteoglycans could be a novel therapy for LSCS and should be analyzed in the future study.

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10. Figure Legends

Figure 1. The thickness of the ligamentum flavum on MRI. A: Lumbar spine on MRI. B: Measuring of the thickness of the ligamentum flavum. On the T1-weighted axial images of the ligamentum flavum through the facet joint, the ligamentum flavum was clearly seen as a low-signal intensity mass just ventral side of the facet joint. The area surrounded by yellow line indicated ligamentum flavum. Arrow head indicated lamina (A). A vertical line to the anterior side of the lamina was made to measure the thickness of the ligamentum flavum (white line). The thickest portion of the ligamentum flavum was measured as thickness (yellow line) (B).

Figure 2. Evaluation of the thickness and the amount of elastic fibers in the ligamentum flavum. A: Elastica-Masson staining of the ligamentum flavum in lumbar disc herniation (LDH), B: lumbar spinal canal stenosis (LSCS). The thickest portion of the ligamentum flavum was measured as a representative thickness. The area in dark purple color within the region of interest (ROI), which corresponds to the ratio of elastic fibers, was calculated. White line indicated a thickness. The area surrounded by broken lines indicated ROI. Scale bars: 500 μm.

Figure 3. Elastic fibers in the ligamentum flavum. A: Elastica-Masson staining of dural side

of the ligamentum flavum in LDH, B: LSCS, C: dorsal side in LDH, D: LSCS. Elastic fibers were significantly smaller in LSCS group compared with the LDH group and that change was evident on the dorsal side of the ligamentum flavum. Dark purple areas indicated elastic fibers (A-D). Scale bars: 200 μm.

Figure 4. High power images of elastic fibers in the ligamentum flavum. A: High power images of Elastica-Masson staining of the ligamentum flavum in LDH, B: LSCS. Elastic fibers were thick and well regulated in the LDH group and sparse, thin, and fragmented in the LSCS group (A and B). Scale bars: 50 μm. Arrows indicated elastic fibers.

Figure 5. Immunostainings of collagen types I and III in the ligamentum flavum. A: Immunoreactivity of collagen type I in LDH, B: dural side of LSCS, C: dorsal side of LSCS, D: collagen type III in LDH, E: dural side of LSCS, F: dorsal side of LSCS. Immunoreactivities of collagen types I and III, which were stained brown, were not significantly different on the dural side of the ligamentum flavum between the LSCS group and the LDH group (A, B, D, E). They were weaker on the dorsal side of the ligamentum flavum in the LSCS group (C and F). Scale bars: 200 μm.

Figure 6. Immunostainings of CTGF and MMP-2 in the ligamentum flavum. A:

Immunoreactivity of CTGF in LDH, B: LSCS, C: MMP-2 in LDH, D: LSCS. Strong immunoreactivities of CTGF and MMP-2 were detected in the LSCS group (A-D). Scale bars: 200 μm. Arrow heads: immunoreactivities of CTGF and MMP-2.

Figure 7. Proteoglycans in the ligamentum flavum. A: alcian blue staining of the ligamentum flavum in LDH, B: LSCS. The intensity of alcian blue staining was significantly stronger in the LSCS group compared with the LDH group. These changes were evident on the dorsal side of the ligamentum flavum (A and B). Arrows indicated the dorsal side of the ligamentum flavum. Scale bars: 500 μm.

Figure 8. Immunostaining of collagen type II in the ligamentum flavum. A: Immunoreactivity of collagen type II in LDH, B: LSCS. Strong immunoreactivity of collagen type II was detected as a spotted area in the LSCS group (A and B). Scale bars: 200 μm. Arrow heads: immunoreactivities of collagen type II.

Figure 9. Immunostainings of decorin and lumican in the ligamentum flavum. A: Immunoreactivity of decorin in LDH, B: LSCS, C: lumican in LDH, D: LSCS. Strong immunoreactivities of decorin and lumican were detected in the LSCS group, especially on the dorsal side (A-D). Scale bars: 200 μm. Arrows indicated the dorsal side of the ligamentum

flavum. Arrow heads indicated immunoreactivities of decorin and lumican.

Figure 10. Immunostainings of versican and osteoglycin in the ligamentum flavum. A: Immunoreactivity of versican in LDH, B: LSCS, C: osteoglycin in LDH, D: LSCS. Strong immunoreactivities of versican and osteoglycin were detected in the LSCS group, especially on the dorsal side (A-D). Scale bars: 200 μm. Arrows indicated dorsal side of the ligamentum flavum. Arrow heads indicated immunoreactivities of versican and osteoglycin.

Figure 11. Immunostainings of aggrecan, biglycan, and fibromodulin in the ligamentum flavum. A: Immunoreactivity of aggrecan in LDH, B: LSCS, C: biglycan in LDH, D: LSCS, E: fibromodulin in LDH, F: LSCS. There were no apparent difference in immunoreactivities of aggrecan, biglycan, and fibromodulin between the two groups (A-F). Scale bars: 200 μm.

Figure 12. Hypothesis of the pathogenesis of thickening of the ligamentum flavum.

Figure 1.

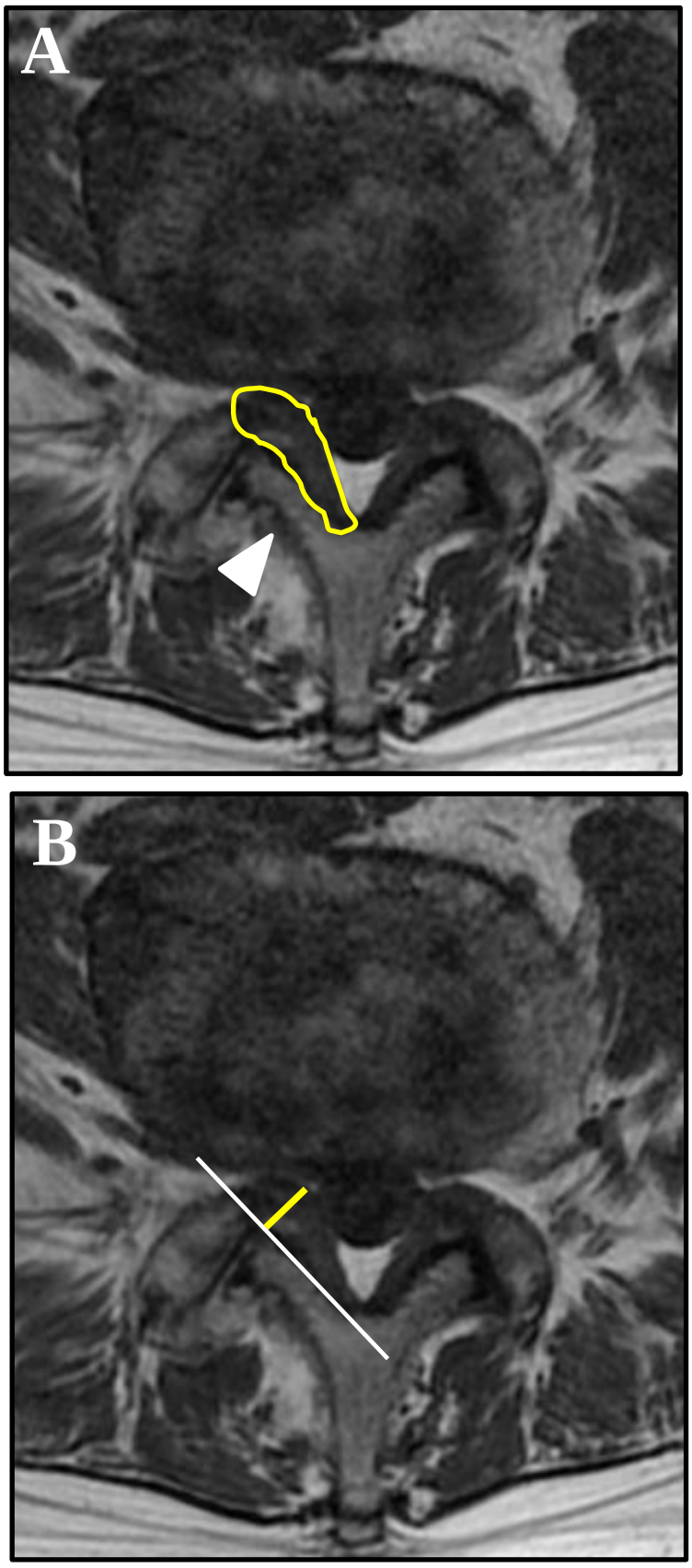
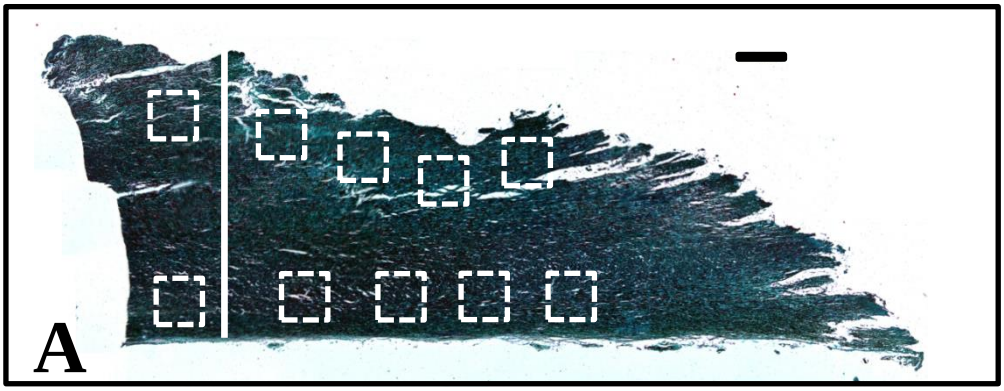


Figure 2.

LDH



LSCS

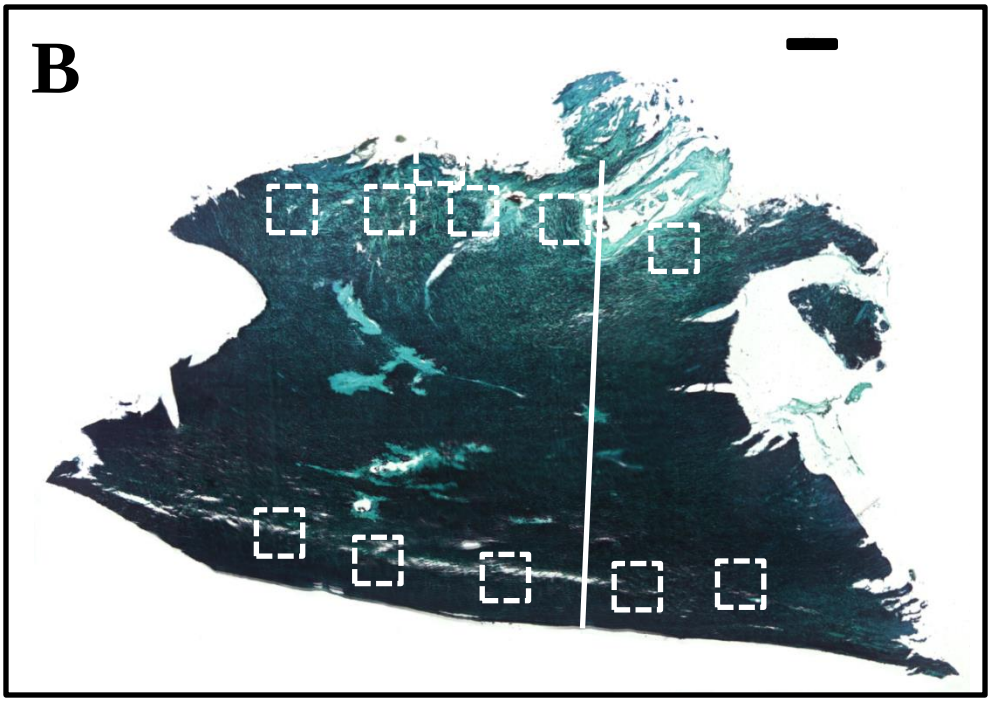
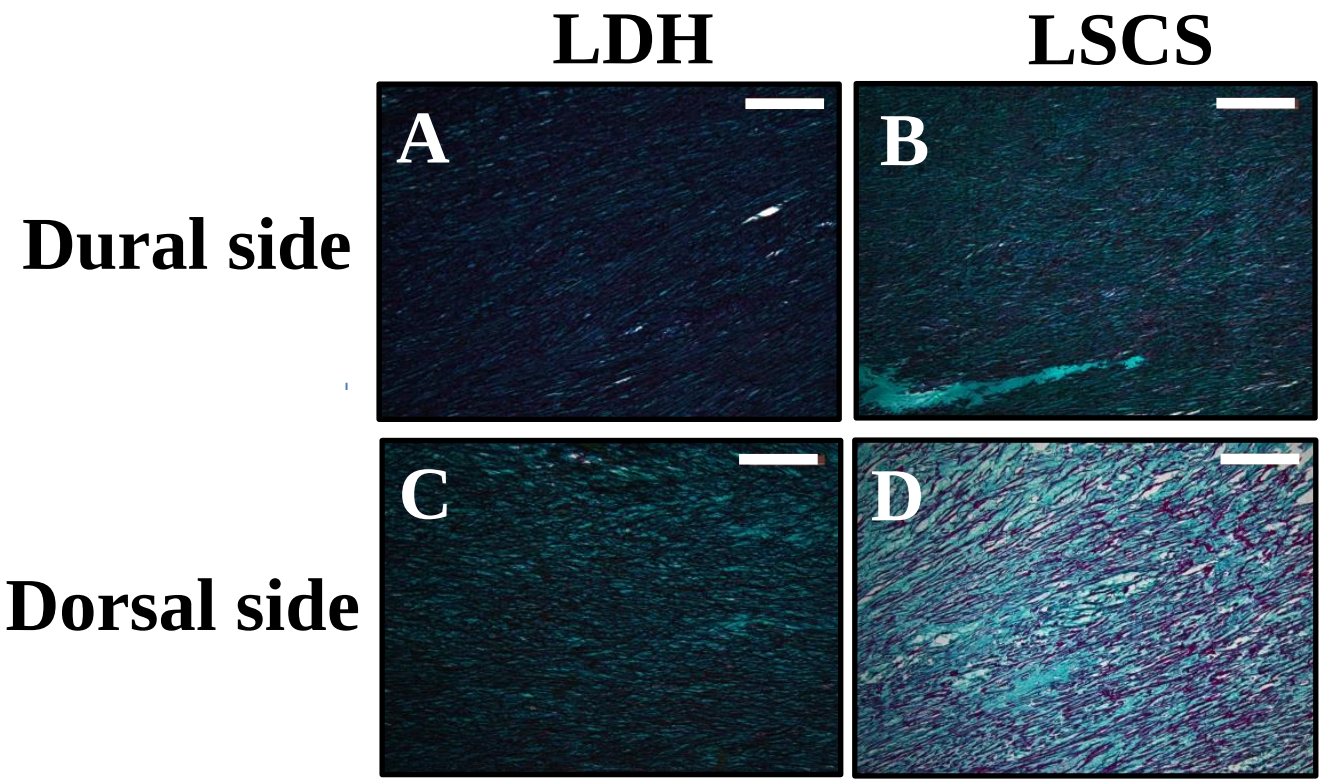


Figure 3.



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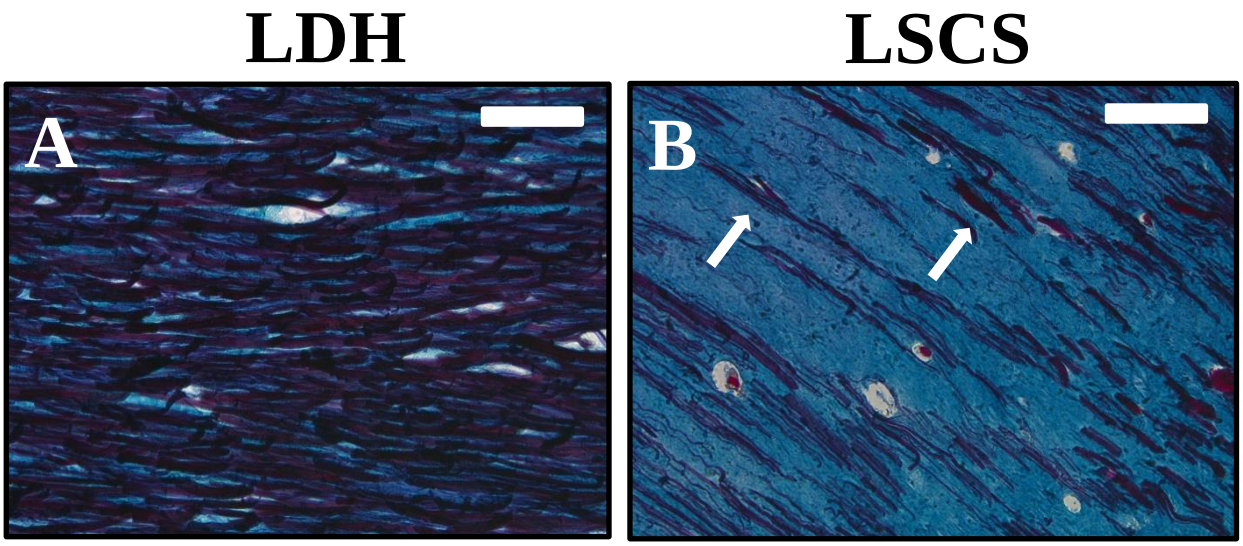

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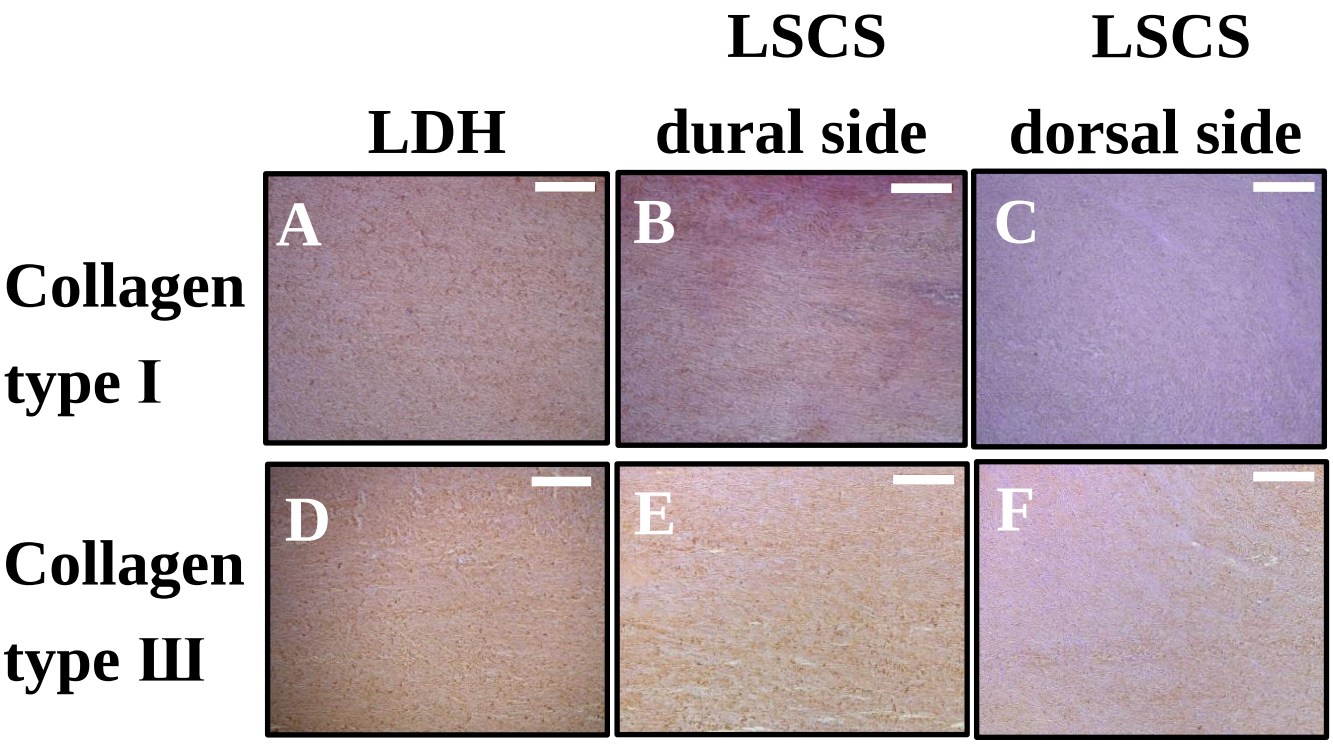

LDH
LSCS
dural side
LSCS
dorsal side
Collagen
type I
Collagen
type III
A
B
C
D
E
F

Figure 6.

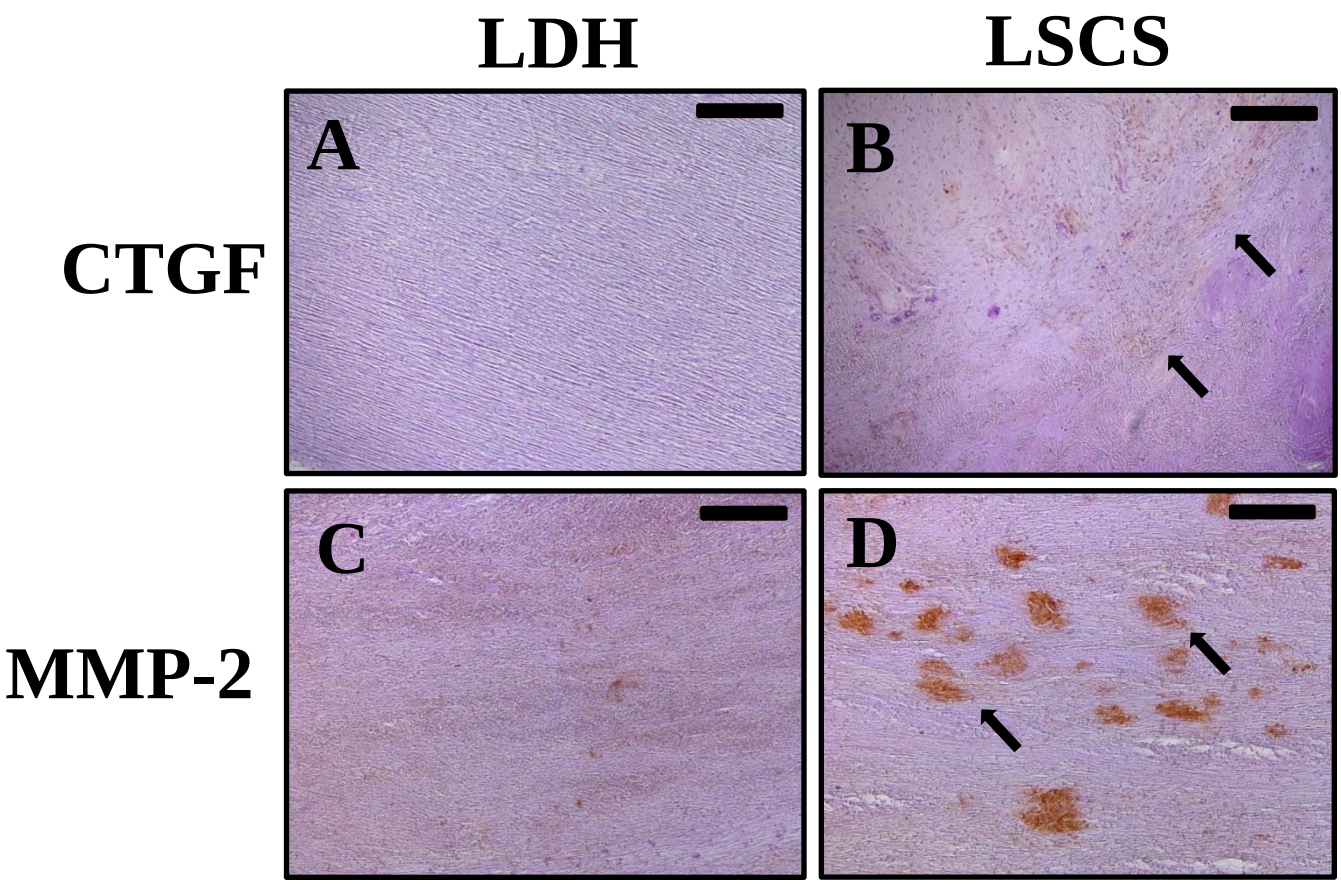
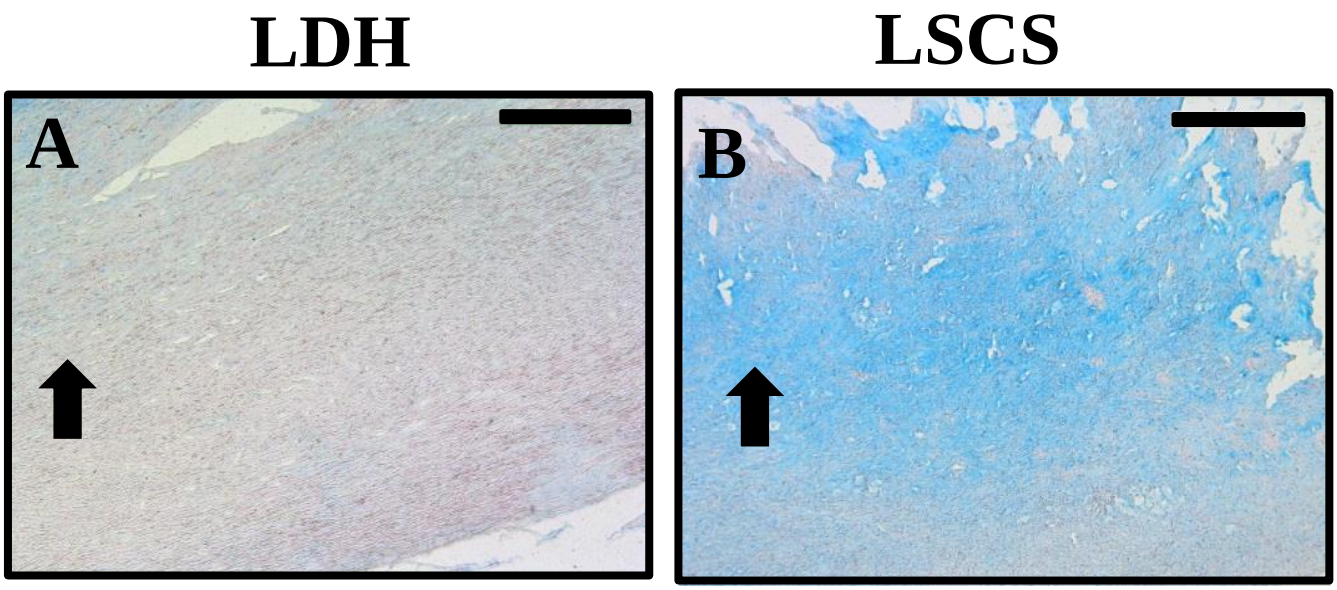


Figure 7.



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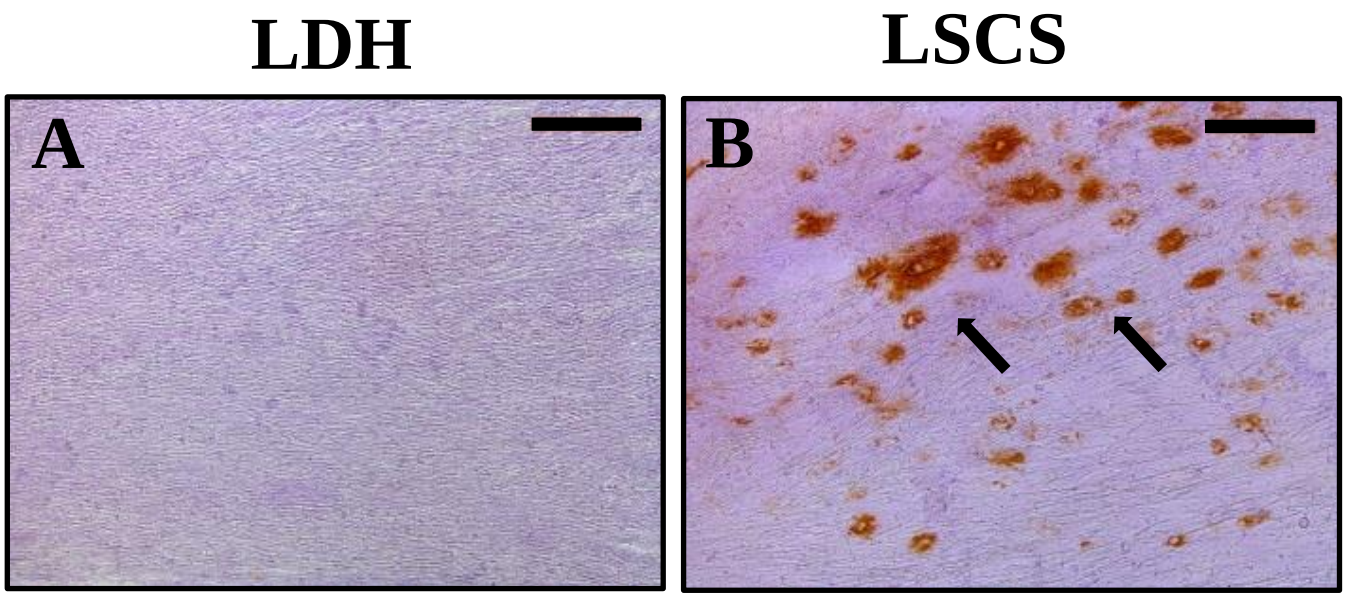


Figure 9.

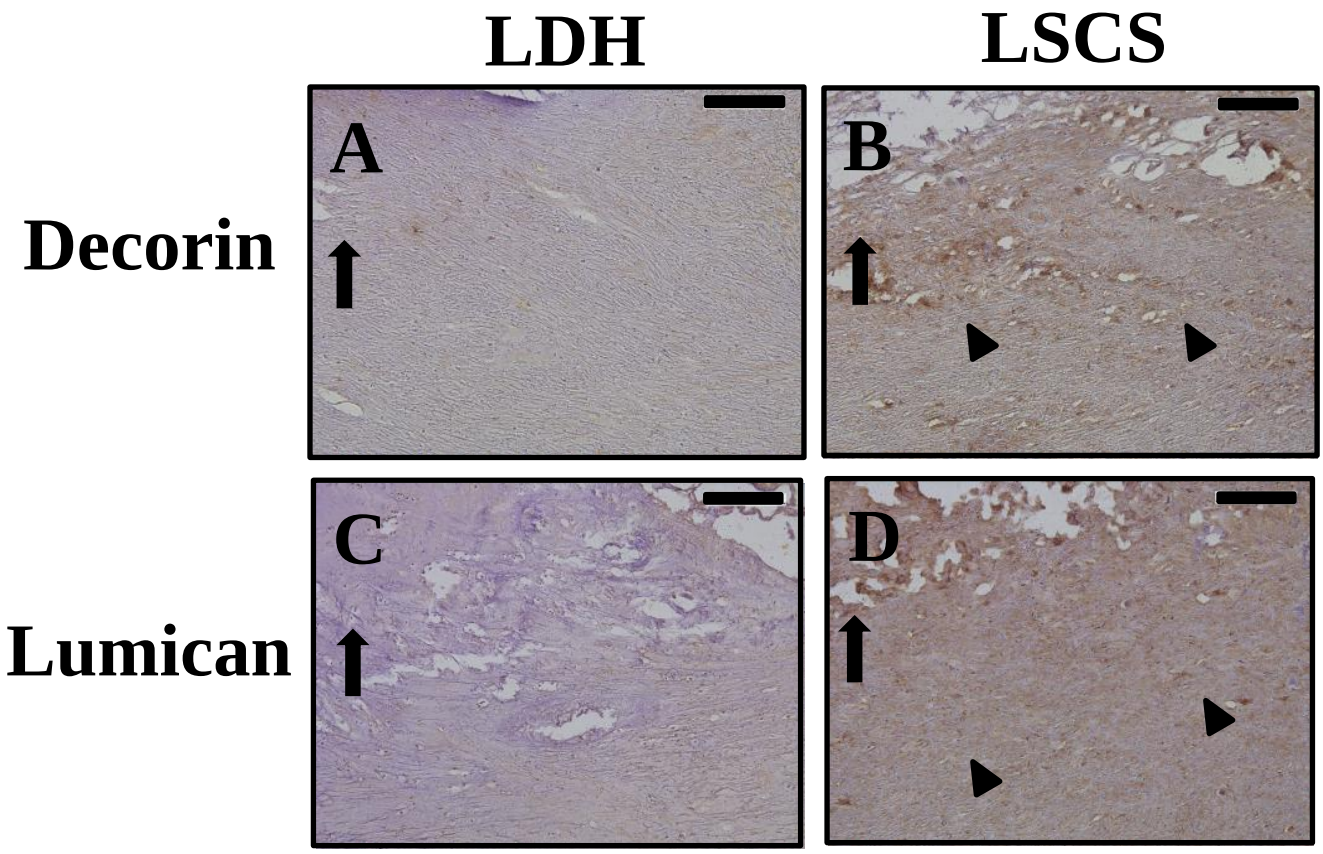
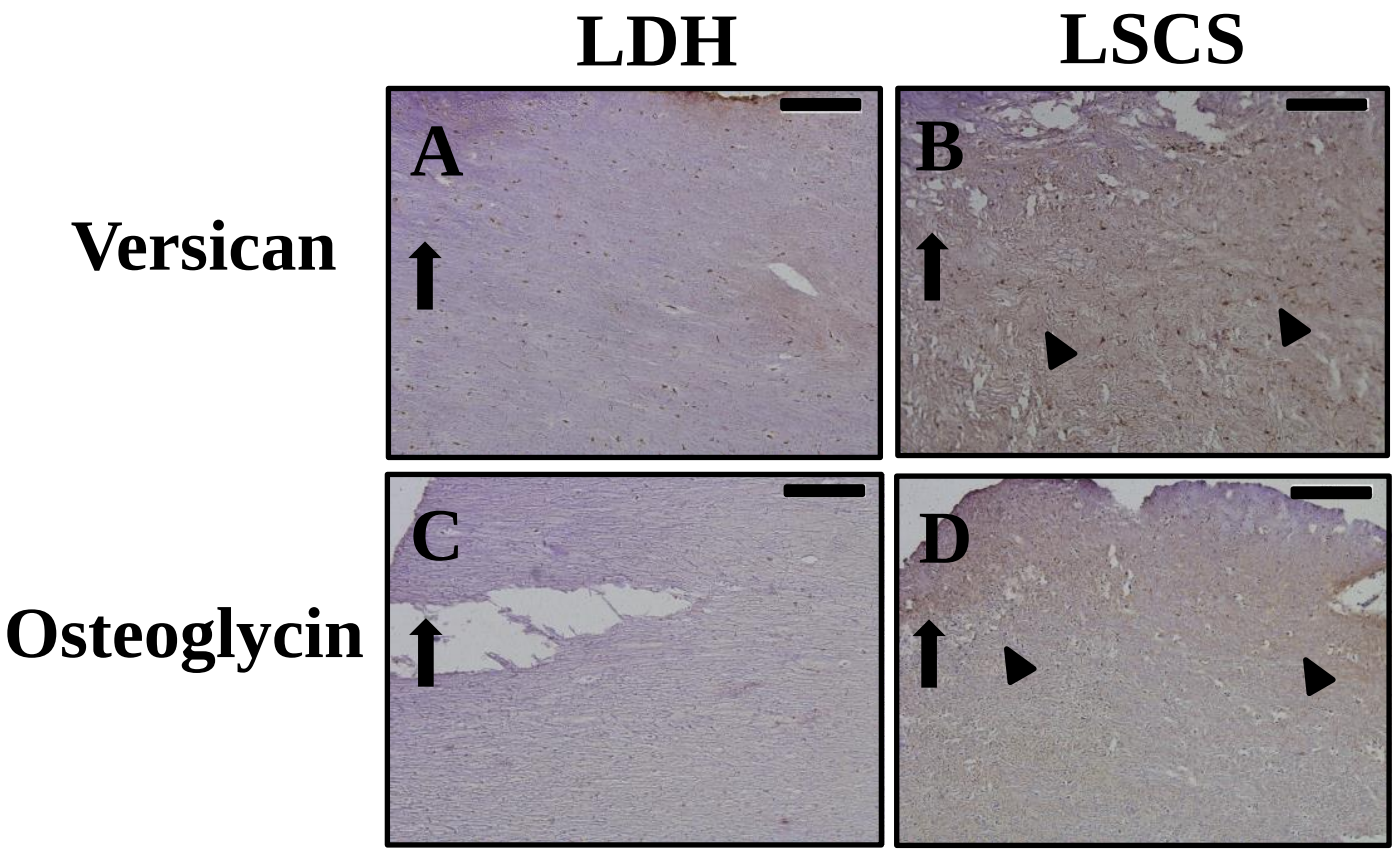


Figure 10.



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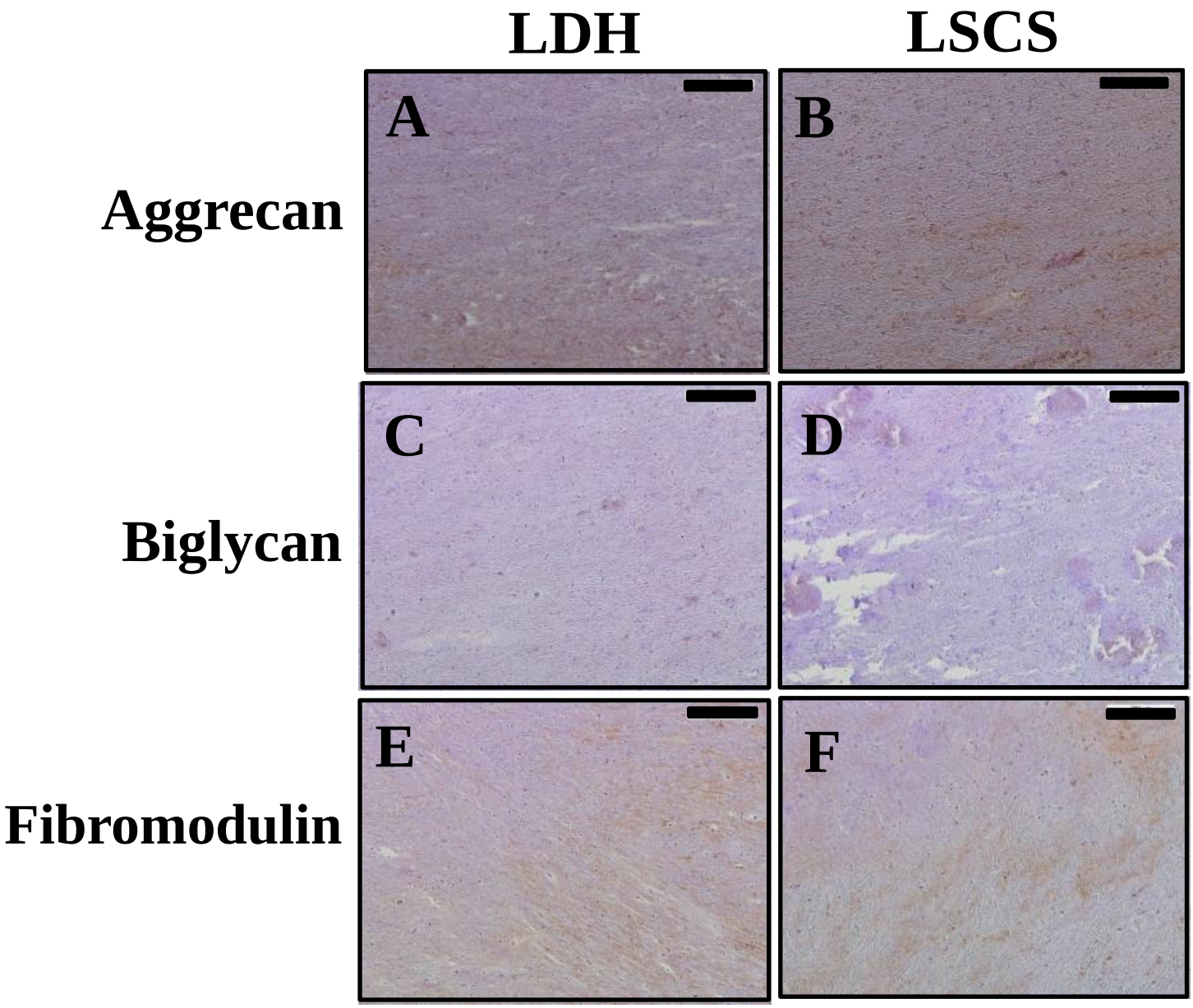

Figure 12.

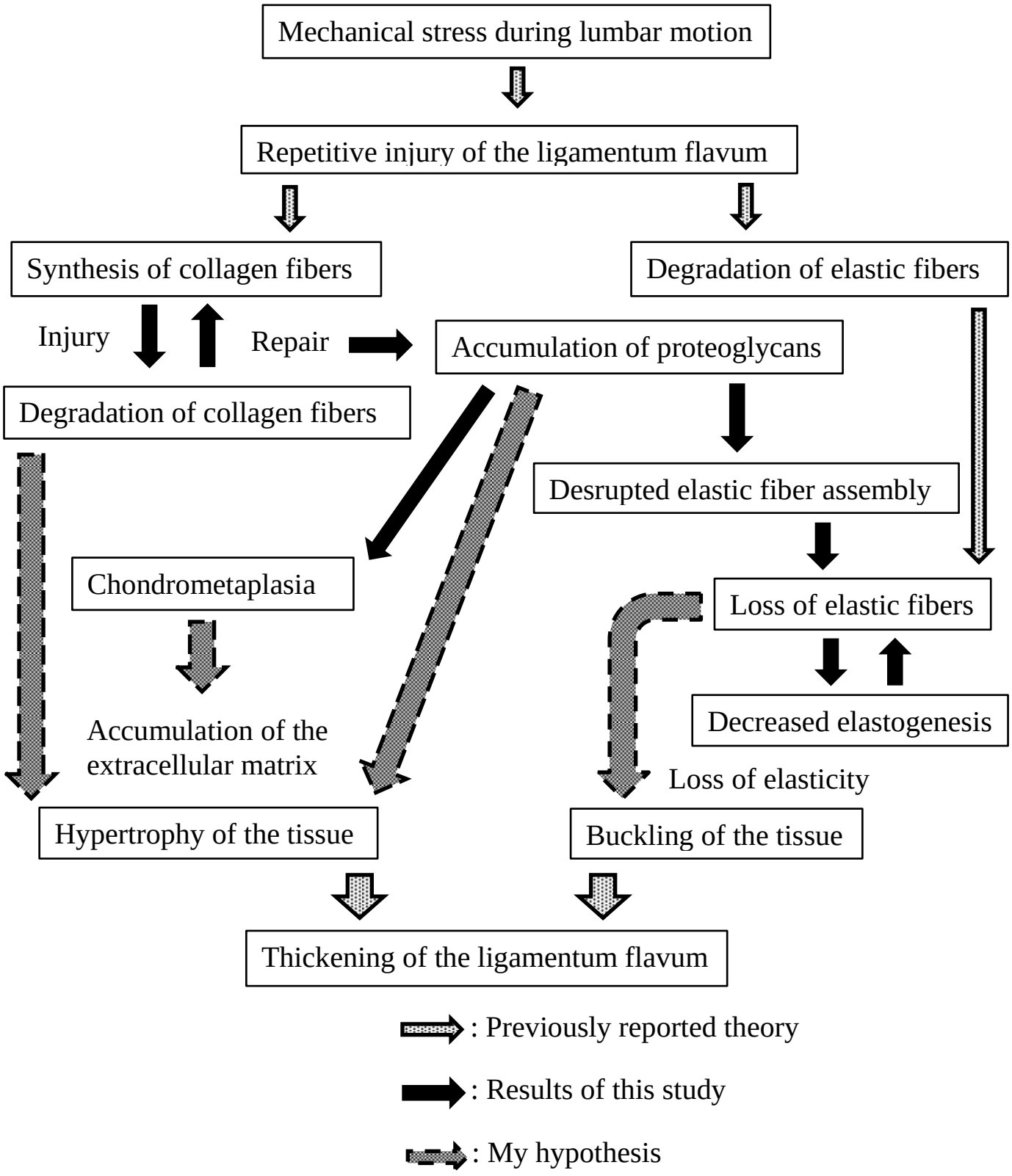
Mechanical stress during lumbar motion
Repetitive injury of the ligamentum flavum
Synthesis of collagen fibers
Degradation of elastic fibers
Injury
Repair
Accumulation of proteoglycans
Degradation of collagen fibers
Desrupted elastic fiber assembly
Chondrometaplasia
Loss of elastic fibers
Decreased elastogenesis
Accumulation of the extracellular matrix
Loss of elasticity
Hypertrophy of the tissue
Buckling of the tissue
Thickening of the ligamentum flavum
: Previously reported theory
: Results of this study
: My hypothesis

Table 1.

Primary antibodies and their dilution.

Antibody	Cat. No.	Products	Dilution
Polyclonal rabbit anti-human collagen type I antibody	ab34710	Abcam	1:200
Monoclonal mouse anti-human collagen type Π antibody	ab3092	Abcam	1:200
Polyclonal rabbit anti-human collagen type ΙΙΙ antibody	ab7778	Abcam	1:400
Polyclonal rabbit anti-human CTGF antibody	ab6992	Abcam	1:200
Polyclonal rabbit anti-human MMP-2 antibody	ab37150	Abcam	1:200
Monoclonal mouse anti-human decorin antibody	ab54728	Abcam	1:800
Polyclonal goat anti-human lumican antibody	AF2846	R & D	1:40
Polyclonal rabbit anti-human versican antibody	ab19345	Abcam	1:200
Polyclonal rabbit anti-human OGN antibody	HPA013132	Sigma	1:100
Monoclonal mouse anti-human aggrecan antibody	Ab3778	Abcam	1:50
Polyclonal goat anti-human biglycan antibody	ab58562	Abcam	1:100
Polyclonal rabbit anti-human fibromodulin antibody	ab81443	Abcam	1:200

Table 2.

Polymerase chain reaction primer sequences.

Gene Name	GenBank	Nucleic acid sequences	
COL1A1	Z74615	Upstream	CGAGGGCCAAGACGAAGACATC
		Downstream	GGGCAGACGGGACAGCACTC
COL1A2	NM_000089	Upstream	TGGTGGTTATGACTTTGGTTACGA
		Downstream	CATGTGCGAGCTGGTTCTT
COL2A1	NM_001844	Upstream	ATCCGGGCAGAGGGCAATAG
		Downstream	ATGATGGGGAGGCGTGAGGT
COL3A1	NM_000090	Upstream	TGAAGGGCAGGGAACAACT
		Downstream	CCGCATAGGACTGACCAAGAT
COL10A1	NM_000493	Upstream	CAGCTTCCCAATGCCGAGTCA
		Downstream	TGCATTTTGTAGGGTGGGGTAGAGTT
EF1α1	NM_001402	Upstream	GGTCGCTTTGCTGTTCGTGAT
		Downstream	AGAGTGGGGTGGCAGGTATTAGG
TGF-β1	NM_000660	Upstream	AGGACCTCGGCTGGAAGTGGAT
		Downstream	AGGCGCCCGGGTTATGCT
αSMA	NM_001613	Upstream	CTATCAGGGGGCACCACTATGT
		Downstream	CTCCGGAGGGGCAATGA
CTGF	NM_001901	Upstream	GAAGAGAACATTAAGAAGGGCAAAAAG
		Downstream	CCGGCAGGGTGGTGGTT
CYR61	NM_001554	Upstream	GCTGCGGCTGCTGTAAGGTCT
		Downstream	CCCACGGCGCCATCAATA
PDGF-BB	NM_002608	Upstream	TAGGGGCATCGGCAGGAGA
		Downstream	ATGGGGGCAATACAGCAAATACC
TNF-α	NM_000594	Upstream	ACCCACGGCTCCACCCTCTC
		Downstream	ACGTCCCGGATCATGCTTTCAG
IL-1β	NM_000576	Upstream	AGGCCGCGTCAGTTGTTGT
		Downstream	CCGGAGCGTGCAGTTCAGT

Table 3.

Polymerase chain reaction primer sequences.

Gene Name	GenBank	Nucleic acid sequences	
MMP-1	NM_002421	Upstream	TGTTCTGGGGTGTGGTGTCTCA
		Downstream	TTCAACTTGCCTCCCATCATTCTT
MMP-2	NM_004530	Upstream	ACGCTGGGCCCTGTCACTC
		Downstream	GGGCCTCGTATACCGCATCA
MMP-3	NM_002422	Upstream	TGACCCAAATGCAAAGAAAGTGA
		Downstream	ATAAAAATGACCGGCAAGATACAGAT
MMP-9	NM_004994	Upstream	TGCCCGGACCAAGGATACAGT
		Downstream	TCAGGGCGAGGACCATAGAGG
MMP-13	NM_002427	Upstream	GAAGACCCCAACCCTAAACATCC
		Downstream	TAAAAACAGCTCCGCATCAACCT
MMP-14	NM_004995	Upstream	CGGGGCATCCAGCAACTTTAT
		Downstream	CCTCACCCGCCAGAACCAG
TIMP-1	NM_003254	Upstream	CCTGGCTTCTGGCATCCTGT
		Downstream	GGCGGGGGTGTAGACGAA
TIMP-2	NM_003255	Upstream	CAAAGCGGTCAGTGAGAAGGAAG
		Downstream	AGGAGGGGGCCGTGTAGATAA
TIMP-3	NM_000362	Upstream	TTGGAGGGCCGATGAGGTAA
		Downstream	CTGGGCGGCCGAGTGATA
ADAMTS4	NM_005099	Upstream	GCGGAGGGGACGGTTCT
		Downstream	TGTGGGGGAGGGCATCA
ADAMTS5	NM_007038	Upstream	CAAGAACGCTGCCACCACAC
		Downstream	GGAGGCCATCGTCTTCAATCA
SOX9	NM_000346	Upstream	GCGGAGCTCGAAACTGACTG
		Downstream	TACCGCGGCGAGCACTTAG

Table 4.

Polymerase chain reaction primer sequences.

Gene Name	GenBank	Nucleic acid sequences	
elastin	NM_001081753	Upstream	GGACCCCTGACTCACGACCTC
		Downstream	TGGCCGCTCCCCTCTTGT
fibrillin-1	NM_000138	Upstream	TGCCCTGGATGGAAAACCTTAC
		Downstream	CCGGCAAATGGGGACAATAC
fibrillin-2	NM_001999	Upstream	GCCCGGCAGCAAACTCAG
		Downstream	AGGGTGGCACAGCATTCAGATT
fibulin-1	NM_001996	Upstream	TCGGGGGCCTCCAAGAAA
		Downstream	CTCCTCGGCAGCGGTCATT
EMILIN-1	NM_007046	Upstream	GCGGGGCCCTGCTCTATG
		Downstream	CGCCCCCGCTGTCTCTTC
DANCE	NM_006329	Upstream	CCACCCCCAGTTCCTATGACA
		Downstream	GACCCCCGCAAACCTAATCTAT
decorin	NM_001920	Upstream	TGCCCACCTGGACACAACAC
		Downstream	GCAGAGCGCACGTAGACACAT
biglycan	NM_001702	Upstream	CTCCTCCAGGTGGTCTATCT
		Downstream	GGTTGTTGAAGAGGCTGATG
lumican	NM_002345	Upstream	CTTCAATCAGATAGCCAGACTGC
		Downstream	AGCCAGTTCGTTGTGAGATAAAC
fibromodulin	NM_002023	Upstream	TGCTGGAATTCCCAACTTCCTCACG GCCATGT
		Downstream	CTCGAGCGGCCGCAACTCATTGATC CTATTGCCT
osteoglycin	NM_033014	Upstream	CCCTGGAATCCGTGCCTCTT
		Downstream	TGCGGTCCCGGATGTAACTG
aggrecan	NM_013227	Upstream	GCCTGCGCTCCAATGACTC
		Downstream	TGGCAATGATGGCACTGTTCT
versican	NM_004385	Upstream	TGGAATGATGTTCCCTGCAA
		Downstream	AAGGTCTTGGCATTTTCTACAACAG

Table 5.

Ratio of elastic fibers.

(%)			
	LDH	LSCS	P
Dural side	72.44 ± 4.73	64.07 ± 9.47	0.018
Dorsal side	68.48 ± 7.08	49.66 ± 7.16	0.00074

Table 6.

Gene expressions of elastogenesis in the ligamentum flavum.

		Mean ± SD	P
elastin	LSCS	0.74 ± 0.37	0.23
	LDH	1.44 ± 1.55	
fibrillin-1	LSCS	1.87 ± 2.62	0.55
	LDH	1.59 ± 1.36	
fibrillin-2	LSCS	0.59 ± 0.31	0.016
	LDH	1.18 ± 0.70	
EMILIN-1	LSCS	0.85 ± 0.48	0.19
	LDH	1.15 ± 0.65	
fibulin-1	LSCS	7.04 ± 11.44	0.42
	LDH	4.14 ± 7.07	
DANCE	LSCS	0.81 ± 0.23	0.036
	LDH	1.05 ± 0.35	

Table 7.

Fibrotic and inflammatory gene expressions in the ligamentum flavum.

		Mean ± SD	P
COL1A1	LSCS	1.40 ± 1.63	0.54
	LDH	1.01±0.41	
COL1A2	LSCS	2.88 ± 2.81	0.023
	LDH	1.09 ± 0.46	
COL3A1	LSCS	14.18 ± 20.01	*0.047*
	LDH	4.21 ± 7.61	
TGF-β	LSCS	1.47 ± 0.86	0.38
	LDH	1.09 ± 0.43	
CTGF	LSCS	1.70 ± 0.78	0.043
	LDH	1.11 ± 0.54	
PDGF-BB	LSCS	2.27 ± 2.08	0.21
	LDH	1.57 ± 1.63	
α-SMA	LSCS	2.46 ± 2.71	0.084
	LDH	1.54 ± 1.67	
CYR61	LSCS	4.16 ± 3.52	0.012
	LDH	1.35 ± 1.25	
TNF-α	LSCS	0.78 ± 0.59	0.17
	LDH	1.42 ± 1.13	
IL1-β	LSCS	0.78 ± 0.44	0.17
	LDH	1.35±1.01	

Table 8.

Gene expressions of MMPs and TIMPs in the ligamentum flavum.

		Mean ± SD	P
MMP-1	LSCS	1.09 ± 0.54	0.56
	LDH	1.37 ± 1.04	
MMP-2	LSCS	2.70 ± 1.81	0.039
	LDH	1.38 ± 1.10	
MMP-3	LSCS	1.10 ± 0.83	0.48
	LDH	1.08 ± 0.38	
MMP-9	LSCS	1.63 ± 1.75	0.25
	LDH	1.21 ± 1.27	
MMP-13	LSCS	7.99 ± 11.54	0.51
	LDH	3.46 ± 3.91	
MMP-14	LSCS	2.64 ± 3.56	0.34
	LDH	1.32 ± 0.82	
TIMP-1	LSCS	2.26 ± 2.10	0.076
	LDH	1.07 ± 0.72	
TIMP-2	LSCS	4.25 ± 2.20	0.044
	LDH	2.37 ± 2.25	
TIMP-3	LSCS	1.59 ± 1.73	0.50
	LDH	2.07 ± 1.99	

Table 9.

Gene expressions of chondrogenesis and proteoglycans in the ligamentum flavum.

		Mean ± SD	P
COL2A1	LSCS	1.99 ± 1.42	0.043
	LDH	0.88 ± 0.43	
COL10A1	LSCS	0.72 ± 0.47	0.11
	LDH	1.22 ± 0.82	
SOX9	LSCS	1.01 ± 0.90	0.30
	LDH	1.67 ± 1.54	
ADAMTS4	LSCS	0.92 ± 0.74	0.54
	LDH	1.03 ± 0.73	
ADAMTS5	LSCS	0.80 ± 0.47	0.093
	LDH	1.22 ± 0.67	
aggrecan	LSCS	1.05 ± 1.21	0.30
	LDH	1.24 ± 0.84	
decorin	LSCS	3.33 ± 2.38	0.020
	LDH	1.25 ± 1.19	
biglycan	LSCS	1.64 ± 0.96	0.11
	LDH	1.05 ± 0.73	
lumican	LSCS	3.91 ± 4.02	0.02
	LDH	1.38 ± 1.33	
fibromodulin	LSCS	2.23 ± 2.17	0.25
	LDH	0.99 ± 0.81	
versican	LSCS	11.50 ± 12.43	0.02
	LDH	2.98 ± 3.76	
osteoglycin	LSCS	4.34 ± 5.70	0.048
	LDH	2.07 ± 2.39	

Table 10.

Increased gene profiles in microarray analysis.

GenBank	Gene Name	Description
NM_001190709	COL11A1	Homo sapiens collagen, typeXI, alpha1
NM_033150	COL2A1	Homo sapiens collagen, typeΠ, alpha1
NM_130830	LRRC15	Homo sapiens leucine rich repeat containing 15
NM_000624	SERPINA5	Homo sapiens serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
NM_014057	OGN	Homo sapiens osteoglycin
NM_001935	DPP4	Homo sapiens dipeptidyl-peptidase 4
NM_003882	WISP1	Homo sapiens WNT1 inducible signaling pathway protein 1
NM_000393	COL5A2	Homo sapiens collagen, typeV, alpha 2
NM_001252	CD70	Homo sapiens CD70 molecule
NM_152282	ACPL2	Homo sapiens acid phosphatase-like 2
NM_212474	FN1	Homo sapiens fibronectin 1
NM_000089	COL1A2	Homo sapiens collagen, typeI, alpha2

Table 11.

Decreased gene profiles in microarray analysis.

GenBank	Gene Name	Description
NM_000432	MYL2	Homo sapiens myosin, light chain 2, regulatory, cardiac, slow, mRNA
NM_001824	CKM	Homo sapiens creatine kinase, muscle, mRNA
NM_001100	ACTA1	Homo sapiens actin, alpha 1, skeletal muscle, mRNA
NM_144994	ANKRD23	Homo sapiens ankyrin repeat domain 23, mRNA
NM_003476	CSRP3	Homo sapiens cysteine and glycine-rich protein 3 (cardiac LIM protein), transcript variant 1, mRNA
NM_203377	MB	Homo sapiens myoglobin, transcript variant 2, mRNA
NM_016599	MYOZ2	Homo sapiens myozenin 2, mRNA
NM_001890	CSN1S1	Homo sapiens casein alpha s1 (CSN1S1), transcript variant 1, mRNA
NM_000257	MYH7	Homo sapiens myosin, heavy chain 7, cardiac muscle, beta, mRNA
NM_003279	TNNC2	Homo sapiens troponin C type 2 (fast), mRNA
NM_003673	TCAP	Homo sapiens titin-cap (telethonin), mRNA
NM_003280	TNNC1	Homo sapiens troponin C type 1 (slow), mRNA
NM_001126132	TNNT1	Homo sapiens troponin T type 1 (skeletal, slow), transcript variant 2, mRNA
NM_001927	DES	Homo sapiens desmin, mRNA
NM_001976	ENO3	Homo sapiens enolase 3 (beta, muscle), transcript variant 1, mRNA
NM_003282	TNNI2	Homo sapiens troponin I type 2 (skeletal, fast), transcript variant 1, mRNA
NM_020349	ANKRD2	Homo sapiens ankyrin repeat domain 2 (stretch responsive muscle), transcript variant 1, mRNA
NM_053044	HTRA3	Homo sapiens HtrA serine peptidase 3, mRNA
NM_001231	CASQ1	Homo sapiens calsequestrin 1 (fast-twitch, skeletal muscle), nuclear gene encoding mitochondrial protein, mRNA
NM_000037	ANK1	Homo sapiens ankyrin 1, erythrocytic, transcript variant 3, mRNA
NM_002475	MYL6B	Homo sapiens myosin, light chain 6B, alkali, smooth muscle and non-muscle, transcript variant 2, mRNA
NM_004102	FABP3	Homo sapiens fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor) (FABP3), mRNA