INTRODUCTION
Ossification of the ligamentum flavum (OLF) progresses insidiously and could cause serious spinal cord compromise (1). Several studies have proposed the possible roles of mechanical, metabolic and genetic factors in the development of OLF, although exact pathomechanism of spinal ligament ossification is still unknown. In histological aspect, enchondral ossification contributed to the ossified process (2). Our previous studies indicated that cell differentiation in the ossification front were differed each OLF case (3), and suggesting these process were regulated under cytokines, growth factors, or signal transduction.

The present study used cultured ligamentum flavum cells under cyclic tensile stress to test the hypothesis that the cell signaling, such as β-catenin pathway (4), plays a role in modulation of chondrocyte and osteoblast differentiation.

PATIENTS AND METHODS
We harvested specimens from 71 patients (40 men, 31 women; average age, 68.7 years) who underwent posterior decompression for symptomatic thoracic OLF. Furthermore, specimens from six patients were used for cell culture in the present study (OLF group; 3 men, 3 women; average age, 71.0 years). Samples of non-ossified ligamentum flavum (NON-OLF group) obtained from six patients who underwent surgeries for other causes were used as control (4 men, 2 women; average age 69.8 years).

Sections were investigated by HE, Elastic van Gieson, and Safranin O stain. Using real-time RT-PCR, we analyzed the mRNA expression levels of β-catenin, Runx2, Sox9, Osterix, Osteopontin and TGF-β in cultured OLF cells subjected to cyclic tensile stress. Cyclic tensile stress was produced by Flexercell® FX-3000. Based on previous study, we set the tensile stress for 10-second cycles of 120% elongation and 10-second of relaxation (5). The expressions of these factors were examined in cultured cell and decalcified paraffin sections by immunohistochemistry.

RESULT
The arrangement of elastic fiber bundles was uniform with collagen fibers in control samples under the microscopy. In OLF samples, the elastic fiber revealed irregular arrangement and/or fragmented in small diameter with increment of thick bundle of collagen fibers. Ossification fronts became wider showing significant irregularities with several disruptions in the calcification line. In addition, a significant number of chondrocytes was found around the calcification front (Fig. 1).

Figure 1. Elastic fiber bundles showed significant irregularities and many chondrocytes gathered around the calcification front in OLF case.

Morphologically, the cultured ligamentum flavum cells exhibited a fibroblast-like, spindle-shaped appearance. The viability of the attached OLF cells was 92.4±2.35% and 90.4±2.90% in 24-hour cyclic tensile stress and non-stress experiments, respectively. At each time point, the viability of stressed OLF cells was not significantly different from that of NON-OLF cells (Fig. 2).

At the real-time RT-PCR analysis, mRNA expression levels of β-catenin, Runx2, Sox9, and Osteopontin were significantly higher in OLF cells under no-stress condition. Application of cyclic tensile stress to OLF samples resulted in significant increases in mRNA expression levels of β-catenin, Sox9, and TGF-β1 at 6-12 hours, and Runx2, Osterix, and Osteopontin at 24 hours (Fig. 3). In NON-OLF case, application of cyclic tensile stress significantly increased the mRNA expression levels of Sox9 and TGF-β but not those of β-catenin, Runx2, Osterix and Osteopontin.

DISCUSSION
The process of ossification in spinal ligament was enchondral ossification including clustering of abnormal fibrocartilage or cartilaginous cells. A number of hypertrophic chondrocytes were gathered around calcification front, and these cells might play an important role for the regulation of formation of ossified plaque by secreting the cytokines or growth factors (2, 3). We considered that transcriptional factors, such as Sox9, Runx2 and Mxs2, contribute to regulate the process chondrocytes differentiation, from mesenchymal cells or fibroblast-like cells to mature chondrocytes in a very complex manner. The mRNA expression levels of transcriptional factors and β-catenin signaling were higher under cyclic tensile stress. These results indicate that transient activation of β-catenin signaling might influence the abnormal cell differentiation in the process of OLF.

SIGNIFICANCE
Chondrocyte differentiation at ossification front plays a key role in OLF since they can undergo enchondral ossification. In this study, we revealed that cyclic tensile stress initiated the ossification process through chondrocyte differentiation mediated by the β-catenin signaling pathway.

References

Figure 2. Cultured ligamentum flavum cells from OLF or NON-OLF patients exhibited a fibroblast-like, spindle-shaped appearance. Cyclic tensile stress does not change the viability of cultured cells.

Figure 3. Relative mRNA expression levels under cyclic tensile stress. (compared with each value at 0 hour).