Introduction
Degeneration of lumbar intervertebral disc is thought to be a cause of low back pain. However, its pathomechanism remains unclear. In patients with chronic discogenic low back pain, sensory nerve fibers are reported to be observed even in the inner layer of the annulus fibrosus or nucleus pulposus, where is not innervated in healthy condition. We speculated exposure of the nucleus pulposus (NP) to the outside of the annulus fibrosus (AF) may promote nerve ingrowth into the inner layer of the annulus fibrosus and it may be one of major causes of discogenic low back pain. In the current study, we evaluated characteristic changes in the rats dorsal root ganglion (DRG) cells using double fluorescent immunohistochemistry and retrograde neurotracing method to elucidate the influence of exposure of NP to the afferent nerve fibers innervating the corresponding disc, and assessed the possible relationship between nerve regeneration and pain perception associated with discogenic low back pain.

Materials and Methods
Twenty four adult male Sprague-Dawley rats weighing 200-250g were used in the study. A neurotracer, Fluoro-Gold, was applied to the ventrolateral aspect of L5/6 disc. Subjects included disc-punctured rats whose disc was punctured from ventral aspect with a 23-gauge needle to expose NP after application of F-G (NP group, n=12) and control whose surface of AF was injured limitedly (sham-operated group, n=12). Rats were perfused on day 10, 20, and 30, and Th13-L5 DRGs which were innervating the L5/6 disc were processed for staining. For immunohistochemistry, we examined double fluorescent staining of growth-associated protein 43 (GAP-43) and calcitonin gene-related peptide (CGRP). GAP-43 is a marker for axonal regeneration which we speculated to be induced after exposure of NP, and CGRP is a peptide contained in small-sized neurons in pain perception. These expressions were compared between two groups at each time point. The cross-sectional area of F-G-labeled GAP-43-IR neurons and F-G-labeled both GAP-43- and CGRP-IR neurons was also measured in all rats and their distributions compared.

Results
The percentage of GAP-43-IR neurons in F-G-labeled neurons were significantly higher in NP group than that of sham-operated group on day 10, 20 and 30 (p<0.05, Fig.1). The percentage of CGRP-IR neurons in F-G-labeled GAP-43-IR neurons were significantly higher in NP group from day 10 to 30 (p<0.05, Fig.2). In GAP-43-IR neurons, the percentage of CGRP-IR neurons in F-G-labeled GAP-43-IR neurons were significantly higher in NP group from day 10 to 30 (p<0.05, Fig.2). In GAP-43-IR neurons, the percentage of CGRP-IR neurons in F-G-labeled GAP-43-IR neurons was not changed significantly in sham-operated group. In cell size distribution of F-G-labeled GAP-43-IR neurons, medium and large-sized neurons occupied over 50% on day 10, but the percentage of small-sized neurons increased gradually (Fig.3). Of F-G-labeled GAP-43- and CGRP-IR neurons in NP group, there were about 35% of small neurons on day 10 and 20, and the percentage increased to 60% on day 30 (Fig.4).

Discussion
Previously, it was reported that rats lumbar intervertebral disc is innervated by NGF-dependent CGRP-IR neurons in pain transmission. In the current study, GAP-43 expression remained significant increase until day 30, suggesting that nerve regeneration continued at least 30days. Additionally, the percentage of CGRP-IR neurons in F-G-labeled GAP-43-IR neurons was significantly increased during all duration in NP group. These results may suggest that exposure of NP to the outside of AF induces nerve regeneration and up-regulation of CGRP at least 30 days and it may be one of causes of discogenic low back pain. In cell size distribution, medium and large neurons are up-regulated in both GAP-43 and CGRP expression until day 20, but the percentage of small-sized neurons were increased in day 30. It is still unclear that how long regeneration of small-sized neurons in pain perception in this study, but it is possible that these changes may be a clue of induction of chronic low back pain.