Introduction: RNA interference (RNAi) technology has recently emerged as an important biological strategy for gene silencing. RNAi is a process of sequence-specific, post-transcriptional gene silencing, induced by double stranded RNA (dsRNA). We reported that RNAi mediated by small interference RNA (siRNA) was effective in specific gene silencing in nucleus pulposus cells in vitro(1). We have also established a microbubble-enhanced ultrasound gene therapy technique for intervertebral disc cells in vivo(2). However, to the best of our knowledge, gene silencing mediated by siRNA in nucleus pulposus in vivo has never reported.

In this study we investigated for the first time gene silencing mediated by native unmodified siRNA in nucleus pulposus in vivo. The objectives were: 1) to estimate the efficacy and duration of downregulation using exogenous reporter gene for the intervertebral disc in vivo using this technique and 2) to certify the downregulation of endogenous gene using the same method.

Materials and Methods: To investigate the efficacy and the longevity of RNA interference in nucleus pulposus of rats in vivo, two reporter luciferase plasmids (Firefly and Renilla) were used. These plasmids were cotransfected with siRNA duplex for targeting Firefly luciferase (DHARMACOM) in the experimental group, and nonspecific siRNA (DHARMACOM) in the negative control group. These plasmids and siRNA were mixed with ultrasonography contrast agent (microbubbles) and injected into coccygeal intervertebral discs of Sprague-Dawley rats. The therapeutic ultrasound was irradiated on the surface of the injected discs. Rats were sacrificed 1, 2, 4, 8, and 24 weeks after gene transduction. Harvested nucleus pulposus tissues were used for evaluation of the Dual-Luciferase Reporter Assay System (Promega). The inhibitory effects of Firefly luciferase gene expression generated by siRNA were expressed as normalized ratios between the activities of the Firefly luciferase and the Renilla luciferase gene (internal control).

In addition, to establish the downregulation of the endogenous gene in vivo, a mixture of five siRNAs targeting rat Fas ligand (FasL) and one non-specific siRNA (used for a negative control) were used. Harvested nucleus pulposus tissues 4 weeks after gene transduction were evaluated for FasL mRNA by RNA isolation and real-time quantitative RT-PCR. The normalized amount of each sample was then determined by each GAPDH mRNA sample.

Results: Longevity of downregulation for exogenous gene.

Our results showed that siRNA for Firefly luciferase can dramatically down-regulate the Firefly luciferase gene expression compared with Renilla luciferase. The average inhibitory rate of siRNA group was about 90% compared with the control group (Figure 1). In contrast, both luciferase gene expressions in the negative control group remained unaffected.

The inhibitory effects were maintained for 24 weeks and even after 24 weeks, the inhibitory rate was 74% compared with the control group (Figure 2).

Discussion: Silencing of gene expression by small interfering RNAs (siRNAs) is rapidly becoming a powerful tool for genetic analysis and represents a potential strategy for therapeutic product development. However, according to previous reports, many systems of gene silencing by native unmodified siRNA show major disadvantages such as needing very high amounts of siRNAs. In addition, their effects are transient, remaining only a few weeks at best without any vector based RNAi. To overcome these problems, many other systems have been developed to improve cellular uptake by using chemically modified siRNA such as short hairpin RNA (shRNA) molecules, additional molecules for resistance to nuclease degradation, and various vectors such as plasmid, virus. Despite the advent of modification strategies to improve cellular uptake and bioavailability in vivo, there is still a necessity to test each siRNA independently, which is time consuming and costly, because chemically modified siRNA is uncertain of the inhibitory effect compared with native siRNA.

We previously reported the longevity of RNAi mediated by native unmodified siRNA in vitro. Although the inhibitory effect in nucleus pulposus cells in vitro continued longer than in other cells, it began to decrease after 2 weeks, and at 3 weeks, it had disappeared completely. In contrast, same siRNA in our in vivo model effectively inhibited exogenous gene expression for at least 24 weeks. Furthermore it also successfully inhibited endogenous gene expression in vivo. Based on these results, we found that using unmodified siRNA in this animal model was sufficient for long term downregulation of gene expression.