Comparative study between gene silencing strategies for intervertebral disc degeneration using RNA interference and CRISPR–Cas9 through the selective interference of mTOR signaling

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Disclosures: MR (N), TY (N), NK (N), YT (Surgical Spine, Inc.), YK (N), TT (N), KM (N), HO (N), TM (N), KKuroshima (N), YH (N), RK (N), KKakutani (Surgical Spine, Inc.)

INTRODUCTION: Low back pain is a major global health issue, and intervertebral disc degeneration is recognized as one of the independent causes[1]. Autophagy is an important cell survival mechanism by self-digestion and recycling damaged components under stress, primarily nutrient deprivation[2]. Intracellular signaling governed by a serine/threonine kinase, the mammalian target of rapamycin (mTOR) negatively regulates autophagy as well as controls cell proliferation and protein synthesis in response to nutrients, growth factors, and energy, while stress, DNA damage, and hypoxia suppress this pathway[3]. More specifically, mTOR exists in the two forms: mTOR complexes 1 (mTORC1) comprising the regulatory-associated protein of mTOR (RAPTOR) and 2 (mTORC2) consisting of the rapamycin-insensitive companion of mTOR (RICTOR)[3]. We hypothesized that mTOR signaling would be influential in the intervertebral disc, which is the largest avascular, low-nutrient organ in the body. Our objective was to elucidate roles of mTOR signaling in disc cells during degeneration based on the comparison between gene silencing strategies using the RNA interference and, more recently developed, clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR associated protein 9 (Cas9) systems.

METHODS: Gene silencing was performed using the two methods, RNA interference (RNAi) and CRISPR–Cas9.
(1) RNAi: Selective gene knockdown of the mTOR signaling pathway using small interfering RNA (siRNA)-mediated RNAi was applied to human disc NP cells, obtained from patients undertaking lumbar discectomy or interbody fusion surgery (n = 6). Monolayer cells were cultured in 10% FBS-supplemented DMEM under 2% oxygen. To knockdown specific gene expression in mTOR-signaling components, siRNA against mTOR targeting mTORC1 and mTORC2, RAPTOR targeting mTORC1, or RICTOR targeting mTORC2 was reverse transfected through lipofection for 36 h. Cells after transfection were additionally cultured in 10% FBS-supplemented DMEM for 24 h. Western blotting for mTOR, RAPTOR, and RICTOR was performed to assess successful transfection with RNAi knockdown efficiency. Autophagic flux was also evaluated by Western blotting. Then, transfected cells were additionally cultured in serum-free DMEM with pro-inflammatory interleukin-1 beta (IL-1β) at 10 ng/ml for 24 h. The incidence of apoptosis and senescence as well as the balance of matrix metabolism were examined by Western blotting.
(2) CRISPR–Cas9: Selective gene knockout of the mTOR signaling pathway using the CRISPR–Cas9 system was applied to human disc NP cells, obtained from patients undertaking lumbar discectomy or interbody fusion surgery (n = 6). As in the RNAi experiment, we examined the knockout efficacy of mTOR signaling, levels of autophagy, apoptosis, senescence, and balance of matrix metabolism by Western blotting.
Statistical analysis: One-way analysis of variance with the Tukey-Kramer post-hoc test was used. Statistical significance was set as $p < 0.05$.

RESULTS: Selective, specific suppression in protein expression of mTOR, RAPTOR, and RICTOR was successfully accomplished by both of RNAi knockdown (Fig. 1A) and CRISPR–Cas9 knockout (Fig. 2A). However, the efficiency of transfection was 53.8–55.6% by RNAi but 88.1–89.3% by CRISPR–Cas9, respectively, which reached statistically significant difference ($p < 0.001$). In both treatments, mTOR signaling suppression-mediated induction of autophagy and inhibition of apoptosis, senescence, and matrix catabolism (Fig. 1B, 1C, 1D) were consistently observed, but the suppression of extracellular matrix degradation was the most prominent in the RAPTOR knockout group by CRISPR–Cas9 (Fig. 2B, 2C, 2D). Additionally in time profiles of RAPTOR gene expression of cells 7 days after transfection, the suppression rate was maintained to 81.8% by CRISPR-Cas9 but only 9.5% by RNAi ($p < 0.001$), indicating a significantly longer duration and higher impact of effectiveness in CRISPR-Cas9.

DISCUSSION: We confirmed the involvement and roles of the intracellular mTOR signaling pathway in human intervertebral disc cells surgically obtained, through the successful inactivation of specific mTOR-signaling components by the two types of gene silencing methods, the RNAi and CRISPR–Cas9 systems. Both gene silencing techniques against RAPTOR in disc NP cells suppressed apoptosis and senescence, which is in agreement with previous reports[4,5]. The observed findings of the CRISPR–Cas9 system, providing extensive gene silencing by knockout, would facilitate comprehensive understanding of the mTOR signaling pathway in the disc. On the other hand, RNAi, delivering temporary gene silencing by non-genome editing knockout, is safer and more favorable as gene therapy for non-critical, chronic intervertebral disc degeneration. In conclusion, RAPTOR/mTORC1 is a potential molecular target to protect against the progression of inflammation-mediated intervertebral disc degeneration.

SIGNIFICANCE: Selective gene silencing of RAPTOR/mTORC1 could be a new therapeutic strategy for degenerative, inflammatory disc disease.

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ACKNOWLEDGEMENTS: This work was supported by JSPS KAKENHI Grant Number 21K09323.

FIGURES: