Improved Long-Term Efficiency of RNAi Using Cationized Gelatin Nanospheres: *Raptor* Knockdown Targeting Selective MTORC1 Inhibition as a New Prevention Strategy for Intervertebral Disc Degeneration

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INTRODUCTION: Low back pain is a major global health problem. Intervertebral disc degeneration is one of its independent causes. In intracellular signaling, the mammalian target of rapamycin (mTOR) is a serine/threonine kinase that integrates nutrient, growth factor, energy, and stress inputs. Autophagy, the intracellular degradation and recycling system, is negatively controlled by the mTOR complex 1 (mTORC1) including the regulatory-associated protein of mTOR (RAPTOR). Selective mTORC1 inhibition by RNA interference (RNAi) of *Raptor* small interfering RNA (siRNA) enhances autophagy and Akt through a negative feedback loop, which could exert disc-protective effects. However, *in vivo*, siRNA is not readily internalized into cells as the native form. The use of virus vectors and lipofection methods has a considerable safety issue. Similarly, irreversibility of genome editing poses a risk of critical side effects, including genetic mutation, cellular dysfunction, and tumorigenesis. In addition, repeated punctures for local injection expedite disc degeneration. Therefore, more efficient, prolonged, and safer gene-therapeutic approaches are desirable. We previously developed an alternative RNAi technology using cationized gelatin nanospheres (cGNS)[1]. However, clinical translation of cGNS was not the focus of prior studies. Here we show for the first time that *in vitro* RNAi transfection of *Raptor* siRNA using cGNS successfully provided a sustained knockdown of RAPTOR protein and induction of autophagy in rat intervertebral disc cells, compared with a conventional lipofection technique. We further clarified that *in vivo* intradiscal cGNS injection achieved a long-term retention of *Raptor* siRNA within the cell, leading to the mitigation of radiological, histological, biological, and biochemical disc degeneration.

METHODS: cGNS were synthesized by introducing spermine into gelatin (isoelectric point 9.0, molecular weight 99,000 Da) and cross-linking after coacervation. *Raptor* siRNA was incorporated at 20 pmol per 1 μg of cGNS ($cGNS_{Rap}$). As a comparator, lipofection (Lipofectamine™ RNAiMAX *in vitro* and Invivofectamine™ *in vivo*) incorporating *Raptor* siRNA was prepared ($Lipo_{Rap}$). ***In-Vitro* study ($n = 16$):** (1) Disc nucleus pulposus (NP) cells harvested from 12-week-old male Sprague-Dawley rats were transfected with $cGNS_{Rap}$ or $Lipo_{Rap}$. Cytotoxicity was assessed by WST-8 assay. (2) Alexa Fluor 555-labeled *Raptor* siRNA was administrated to compare time-course intracellular uptake between $cGNS_{Rap}$ and $Lipo_{Rap}$. ***In-Vivo* study ($n = 64$):** (1) *Raptor* siRNA (2 μl, 60 pmol/μl) formulated as $cGNS_{Rap}$ or $Lipo_{Rap}$ was injected into rat coccygeal discs. Intradiscal siRNA distribution, cellular uptake, and intracellular retention were evaluated by cryosections at sequential time points. (2) Cy5-labeled cGNS were injected into rat tail discs. Intradiscal cGNS biodegradability was monitored longitudinally using an *in vivo* imaging system. (3) While $cGNS_{Rap}$ were injected into Co8/9 and Co11/12 discs, cGNS of negative control siRNA ($cGNS_{ctrl}$) were applied to Co9/10 and Co12/13 discs (all 2 μl, 120 pmol siRNA). Co13/14 disc also received injection of saline at 2 μl as the sham control. At 7 d after injection, temporary static compression at 1.3 MPa was applied for 24 h to Co8/9 and Co9/10 discs. After compression release, protective effects of $cGNS_{Rap}$ and $Lipo_{Rap}$ were assessed by serial radiographic analysis using the disc height index (DHI) and histomorphological safranin orange staining evaluation with a 16-point grading system[2]. (4) At 28 d after mechanical stress, protein expression was examined by immunofluorescence for RAPTOR, p16/INK4A, and TUNEL and immunohistochemistry for COL2A1, aggrecan, and aggrecan neoepitopes [DIPEN, ARGSV]).

RESULTS: *In-Vitro* study: (1) Maximum non-toxic concentration of siRNA was 400 pmol/ml using $cGNS_{Rap}$, compared with 60 pmol using $Lipo_{Rap}$, allowing 6.7-fold higher administration (Fig. 1A). (2) siRNA and cGNS were co-localized in the cytoplasm, and the percentage of siRNA-positive cells were significantly higher in $cGNS_{Rap}$ than $Lipo_{Rap}$ at all time points (all $p < 0.0500$) (Fig. 1B). ***In-Vivo* study:** (1) Following intradiscal injection, the percentage of siRNA-positive cells was significantly higher in $cGNS_{Rap}$ than $Lipo_{Rap}$ at all time points ($p < 0.0001$) (Fig. 2A). (2) cGNS gradually decreased in fluorescence intensity, yet 26.1% ± 13.5% of the signal remained at 84 d after injection ($p < 0.0001$) (Fig. 2B). (3) In a rat tail temporary static compression model, discs injected with $cGNS_{Rap}$ maintained significantly higher %DHI compared with $cGNS_{ctrl}$ at 28 (78.9% ± 6.1% vs. 67.2% ± 10.4%, $p = 0.0468$), 56 (80.1% ± 10.2% vs. 61.1% ± 4.5%, $p = 0.0002$), and 84 d after mechanical stress (77.7% ± 4.3% vs. 52.1% ± 9.2%, $p < 0.0001$) (Fig. 2C). Histopathological grading scores were also significantly lower in $cGNS_{Rap}$-treated discs at 84 d after compression (3.5 [2–5] vs. 8.0 [7–12], $p = 0.0142$) (Fig. 2D). (4) At 28 d after mechanical loading, immunofluorescence demonstrated fewer RAPTOR-positive (24.0% ± 5.4% vs. 52.0% ± 7.6%, $p = 0.0155$), TUNEL-positive (16.5% ± 2.5% vs. 26.8% ± 7.0%, $p = 0.0061$), and p16/INK4A-positive cells (24.0% ± 5.4% vs. 52.0% ± 7.6%, $p = 0.0005$) in $cGNS_{Rap}$-treated discs compared with $cGNS_{ctrl}$. Similarly, immunohistochemistry revealed maintained expression of COL2A1 and aggrecan as well as reduced expression of aggrecan neoepitopes, particularly ADAMTS-cleaved ARGSV (163.0% ± 62.8% vs. 381.9% ± 74.1%, $p = 0.0010$) (Fig. 2E).

DISCUSSION: *In vivo*, *Raptor* siRNA incorporating cGNS enabled sustained siRNA retention and effective target RAPTOR protein knockdown, leading to disc height maintenance, reduced degeneration, and protection against apoptosis, senescence, and matrix degradation. These findings suggest the potential of cGNS as a safe and efficient drug delivery system for long-term RNAi therapy in the intervertebral disc, improved relative to conventional lipofection.

SIGNIFICANCE/CLINICAL RELEVANCE: RNAi using cGNS-mediated *Raptor* siRNA delivery offers a minimally invasive strategy for the intervertebral disc protection through selective MTORC1 inhibition, possibly representing a new therapeutic approach for degenerative disc disease.

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