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Progress report: ISSLS Research Grant sponsored by Taisho Pharmaceutical

Dear Research Committee,

First of all, we would like to express our gratitude to ISSLS and particularly to Taisho Pharmaceutical for funding our project entitled "***Towards personalized delivery and activation of GDF-5 for regeneration of the intervertebral disc***". The grant provided us with the opportunity to investigate a cutting-edge gene delivery technology based on CRISPR synergistic activation mediator (SAM). During the course of this project, we validated the ability of CRISPR to activate GDF-5 in human primary nucleus pulposus (NP) cells and showed that CRISPR activation can reverse inflammation-related downregulation of GDF-5. To safely deliver CRISPR SAM into NP cells in clinically relevant manner, we investigated a novel non-viral gene delivery method, which is based on nanovesicles derived from patient's own cells (so-called nanoghosts). Our experiments indicated that nanoghosts prepared from MSCs might be able to deliver functional CRISPR SAM DNA into primary NP cells. As present data can be considered preliminary, the project will continue to verify our investigations and further optimize the activation of endogenous GDF-5.

Dr. Olga Krupkova (OK) benefited enormously from this grant opportunity. The project allowed OK to learn new skills, generate new collaborations with academics and surgeons, and progress towards scientific independence. The collected data constitute a foundation for further grant applications with OK as principal investigator. Upon receiving the ISSLS Research Grant and generating preliminary data, this project was also funded by the *Eurospine* and *Kurt and Senta Herman Foundation*. Notably, we received a prestigious *Swiss Government Excellence Scholarship (2020)* and *IBSA Foundation Fellowship (2020)*, which enables us to employ a full-time postdoctoral researcher (Dr. Anna Gryadunova), who will continue developing this project under the supervision of OK.

We believe we can develop a versatile gene delivery tool with relevance for both basic and translational research. Once optimized, our personalized gene delivery tool can be readily adapted to target any gene and multiple genes in a single cell. The correction of IVD disease-associated phenotypes by regulating multiple genes at once is an attractive future goal and possibly a major step forward in personalized treatments. This tool can be also adapted to deliver other cargos (e.g. drugs), thus a wide range of researchers might also benefit from this strategy in the future.

Sincerely,

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Towards personalized delivery and activation of GDF-5 for regeneration of the intervertebral disc

1. INTRODUCTION

Chronic low back pain (LBP) is a leading cause of disability and the main reason for non-cancer opioid prescriptions[1, 2]. LBP is strongly associated with intervertebral disc (IVD) degeneration[3]. Discogenic LBP is currently treated either by common pain medication or invasive surgeries, with risks of adverse effects, slow recovery, and high rates of reoccurrence[4, 5]. **Despite the large need for cell-based IVD regeneration, such therapy has not been broadly adopted clinically**. The main limitation for the translation of cell-based IVD therapies into clinical practice is the harsh microenvironment of degenerated nucleus pulposus (NP), which negatively affects performance and/or survival of therapeutic (and resident) cells[6-10]. The recent consensus in the field is that therapeutic cells should be preconditioned or engineered to function better in the harsh microenvironment[1].

The regenerative capacity of IVD cells could be boosted by the upregulation of growth and differentiation factor 5 (GDF-5), a factor critical for the maintenance of IVD homeostasis[11]. GDF-5 was shown to induce IVD extracellular matrix (ECM) repair[11-13], which led to several clinical trials on intradiscal application of recombinant GDF-5 (*clinicaltrials.gov*). However, direct application of growth factors has severe limitations such as the need for repeated injections of highly concentrated dose and potential adverse effects[7]. To overcome this limitation, **we propose targeted gene activation using CRISPR synergistic activation mediator (SAM)**[14]. Apart from specific small guide (sg)RNA, CRISPR SAM transcription activation system contains catalytically inactive Cas9 fused with VP64 activation domain and other factors that enhance the transcription of the endogenous target gene (***Fig. 1***). As clinical translation of gene therapy is hindered by safety concerns associated with common gene delivery vectors [15], **we also propose a novel non-viral gene delivery approach.** CRISPRa-GDF-5 DNA will be delivered into IVD cells via nanoghosts (NG), nanosized vesicles derived from patient's own cellular membranes, e.g. from erythrocytes or MSCs[15]. NG are able to deliver various cargos including DNA and show good translation potential in terms of efficiency, biocompatibility, safety, scalability, high yields, and cost-effectiveness[15].

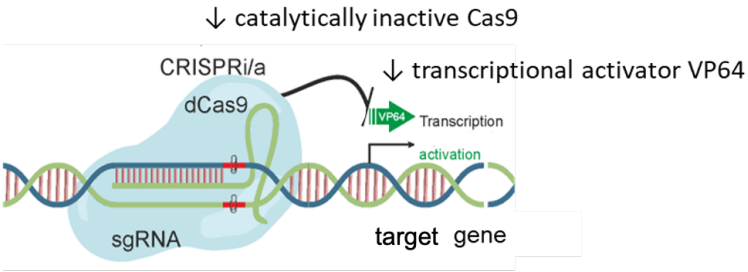


Fig.1. Schematic representation of CRISPR synergistic activation mediator (SAM).

The **goal** of the project is to develop/test a novel non-viral approach that delivers functional CRISPR-GDF-5 DNA into NP cells in a personalized manner. The **novelty** of our approach is personalization designed in two steps (1) via the delivery method (membranes of patient's own cells are used as delivery vehicles) and (2) via patient-specific genome regulation (correction of a particular genetic background and/or disease condition).

Based on our preliminary data and state-of-the-art literature **we hypothesize** that:

(i) CRISPR SAM DNA can activate endogenous GDF-5 in NP cells
(ii) NG can be prepared from autologous MSCs and/or erythrocytes
(iii) NG can deliver large one-vector CRISPR SAM DNA
(iv) CRISPR SAM DNA delivered by NG is biologically functional

Specific Aims of the project are:

(a) To generate and validate CRISPR SAM DNA in NP cells
(b) To generate and characterize nanoghosts
(c) To optimize CRISPR SAM DNA delivery into NP cells
(d) To analyze bioactivity of the system (3D & *ex vivo* models)

2. RESULTS

2.1 Validation of CRISPR SAM GDF-5 DNA

Human NP tissue was collected by an experienced surgeon, with informed consent and ethical approval. Three different small guide RNAs (sgRNA) were tested for their ability to activate GDF-5 in NP cells using established lentiviral vectors. A CRISPR SAM system containing the most efficient sgRNA activated gene/protein expression of GDF-5 in NP cells (***Fig. 2***). The activation of GDF-5 in NP cells was associated with enhanced gene expression of collagen type II, TIMP1 and MMP3 ($p<0.05$), indicating its ability to modulate ECM. Importantly, this CRISPR SAM vector activated GDF-5 also in IL-1b-stimulated NP cells ($p<0.05$) where the expression of GDF-5 is normally inhibited by inflammation, thus supporting the functionality of this approach (***Fig. 2C***).

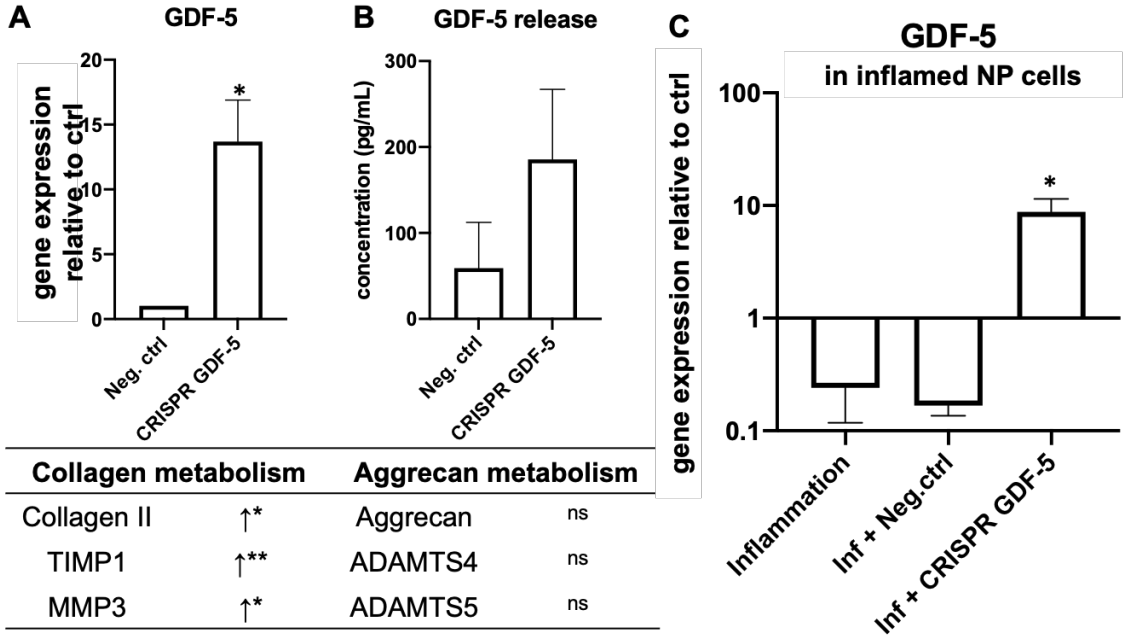
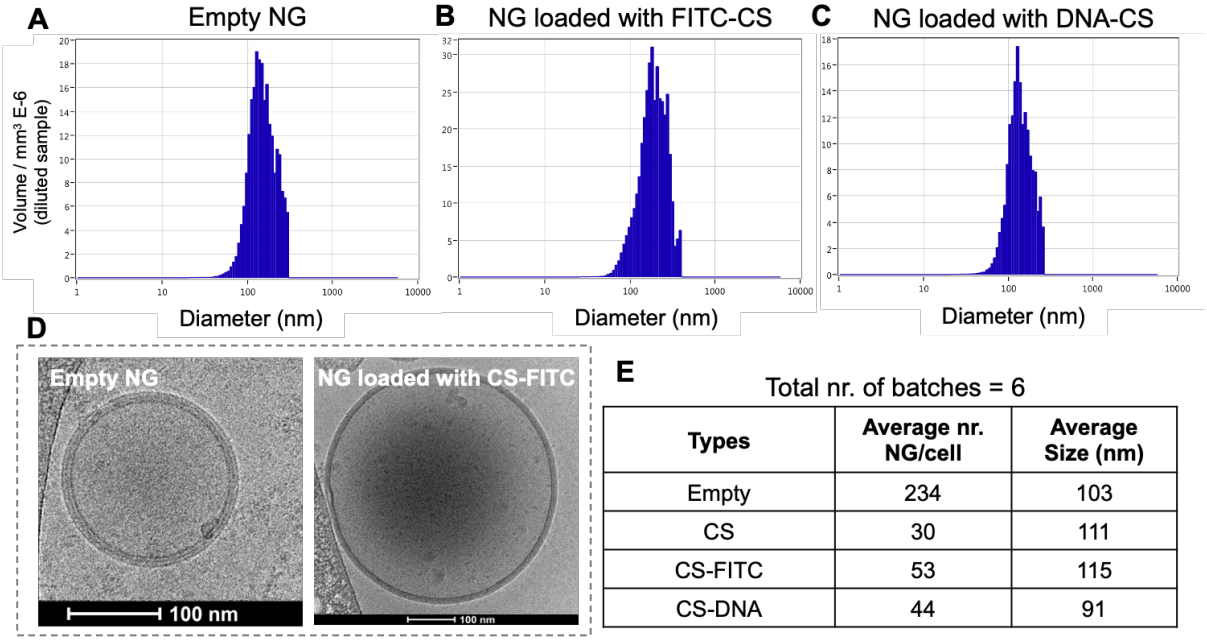


Fig. 2. Validation of CRISPR SAM GDF-5 DNA in NP cells. *NP cells (n = 5) were treated with CRISPR SAM GDF-5 or neg. ctrl lentivectors.* ***(A)*** *GDF-5 gene expression,* ***(B)*** *GDF-5 protein release,* ***(C)*** *Gene expression of GDF-5 in NP cells pre-stimulated with 5 ng/mL IL-1b ($p < 0.05$ ANOVA or t-test vs. ctrl).*

2.2 Generation and characterization of nanoghosts (NG)

NG were prepared from immortalized mesenchymal stromal cells (MSCs)[16] as described before[15]. CRISPR SAM DNA (0.5-50 ug) targeting GDF-5 or non-target (NT) were generated using established protocols and encapsulated into NG. DNA was encapsulated in free form or complexed with chitosan (CS, 10 mg/mL), to enhance its nuclear uptake[17]. Empty NG and NG loaded with FITC-CS and CS alone were prepared as well. NG morphology, concentration and size were determined using Cryo-TEM and nanoparticle tracking analyzer (NTA) (***Fig. 3A-C***). Total 6 batches of NG were generated. NTA analysis revealed the normal distribution of sizes/volumes, with a cut-off at 0.4 µm due to filtration. NG loading was characterized by a darker colour, indicating the presence of a cargo ***Fig. 3D***).



Types	Average nr. NG/cell	Average Size (nm)
Empty	234	103
CS	30	111
CS-FITC	53	115
CS-DNA	44	91

Fig. 3. Generation and characterization of nanoghosts (NG). (A-C) *NTA analysis of* ***(A)*** *empty NG,* ***(B)*** *NG loaded with FITC-chitosan (CS),* ***(C)*** *NG loaded with DNA-CS.* ***(D)*** *Representative images of empty (left) and loaded (right) NG.* ***(E)*** *Average nr. of NG per donor cell and their average size.*

2.3 The effects of nanoghosts (NG) on human NP cells

Next, NG were applied on target NP cells cultured in monolayer. **NG interaction with NP cell membranes** was tested using NG loaded with FITC-CS (200 NG/target cell after 3 hours of treatment (***Fig. A-C***). Resulting 11.3% of FITC-positive cells were found in the NG-treated group (***Fig. 4C***) compared with 0.02-0.08% in control groups, suggesting the potential uptake of NG into NP cells (n = 1). The mechanism of cellular uptake will be further verified in more NP donors. The **non-toxicity of the NG preparations** was tested by MTT assay in NP cells (n = 3) treated with increasing concentration of NG (0-800 NG/cell) (***Fig. 4D***). No negative effects of NG treatment vs. control (PBS) were detected. It is thus possible to select the concentration of up to 800 NG/cell for further testing. The **delivery of DNA into NP cells** (n = 3) was also investigated. Large CRISPR SAM DNA (13.3 kb) that carries mCherry marker was encapsulated into NG either naked or complexed with CS (DNA:CS) and NP cells were treated with these preparations at 200 NG/cell (***Fig. 4E***). Gene expression of mCherry was detected after 4 days, especially in NP cells treated with NG containing DNA:CS ($p<0.05$). The data suggested that large CRISPR DNA delivered by NG can be expressed in NP cells, with CS enhancing its activity. However, no significant regulation of GDF-5 was found in these samples (data not shown). Therefore, our NG CRISPR DNA delivery protocols must be optimized further.

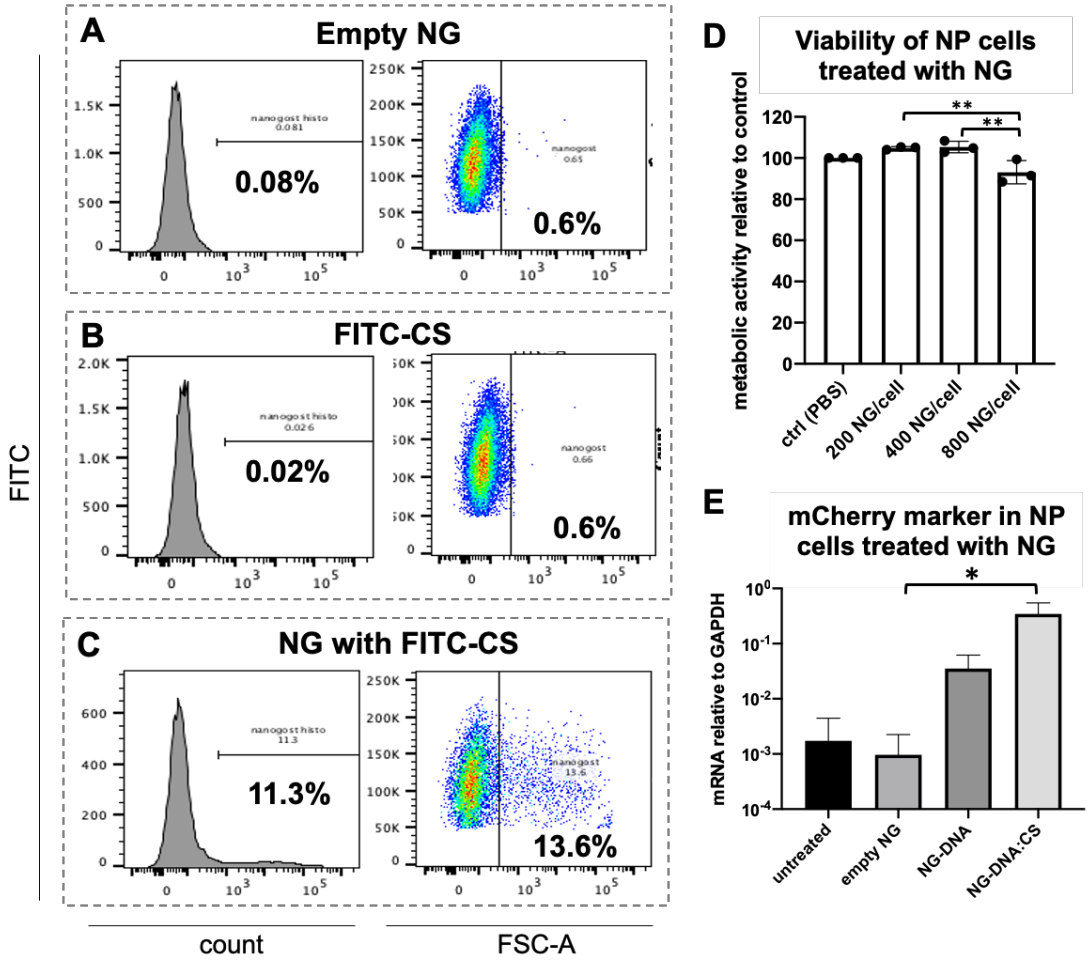


Fig. 4. The effects of nanoghosts (NG) on human NP cells. (A-C) *Cellular uptake was measured by FACS in NP cells (n = 1) treated with* ***(A)*** *empty NG,* ***(B)*** *free FITC-CS and* ***(C)*** *FITC-CS encapsulated in NG.* ***(D)*** *Cell viability was determined by MTT assay in NP cells treated with increasing concentration of NG (n = 3, $p < 0.05$ ANOVA).* ***(E)*** *NP cells were treated with NG containing CRISPR SAM DNA with or without CS, or empty NG. Upregulation of a marker gene mCherry was detected (n = 3, $p < 0.05$ ANOVA).*

3. DISCUSSSION AND FUTURE PERSPECTIVES

During the course of this project, we have validated the activity of CRISPR SAM in human primary NP cells and showed that CRISPR activation can reverse inflammation-related downregulation of GDF-5. Therefore, using this method it might be possible to upregulate endogenous GDF-5 in harsh degenerative conditions normally inhibiting its expression *in vivo*. However, CRISPR SAM DNA is large (>10 kb), which limits its cell delivery and application in regenerative medicine[18-20]. Our experiments indicated that it might be possible to deliver CRISPR SAM DNA using NG prepared from MSCs, as indicated by the expression of the mCherry marker in NP cells. An advantage of NG derived from MSCs is that they may possess unique inflammation-targeting capabilities [33], a feature significant for IVD regeneration. However, we were not yet able to achieve significant upregulation of endogenous GDF-5 using CRISPR SAM delivered by NG. To address this, we will extend our NG preparation protocols (optimization of DNA loading, purification of NG preparation).

We will also investigate the possibility to generate NG from patient's own erythrocytes (nanoerythrosomes, NE), a readily available cell type without its own DNA. This could significantly

broaden the applicability of the proposed method. In collaboration with the Stoyanov group (Nottwil, CH), we have already developed a method for the rapid preparation of NE (*https://doi.org/10.2147/DDDT.S258368*). This method will be also employed in this project for NG-based CRISPR SAM GDF-5 activation.

The presented experiments can be considered preliminary, thus will be repeated and verified in multiple NP donors (at least n = 10). The bioactivity of NG-encapsulated CRISPR DNA will be further analyzed in 3D cultures and in whole bovine IVD *ex vivo* model[21, 22].

4. SIGNIFICANCE

This is the first study that tests the use of NGs for the delivery of large CRISPR DNA in the field of musculoskeletal regeneration. The concepts investigated in this project are highly relevant to personalized medicine. The applicability of gene therapy in patients is still rather narrow, due to the lack of suitable gene delivery methods. If successful, the project will bring **(1)** a non-viral gene delivery method for regenerative medicine, **(2)** a proof of concept that NG can deliver large CRISPR DNA, **(3)** a basis for allele-specific genome regulation and correction of disease phenotypes, **(4)** findings that could be extended to other cell types and diseases, and **(5)** an interdisciplinary approach (clinical and academic collaboration) to ensure the translational potential of our findings.

5. REFERENCES

1. Smith, L. J.; Silverman, L.; Sakai, D.; Le Maitre, C. L.; Mauck, R. L.; Malhotra, N. R.; Lotz, J. C.; Buckley, C. T., Advancing cell therapies for intervertebral disc regeneration from the lab to the clinic: Recommendations of the ORS spine section. *JOR Spine* **2018,** *1* (4), e1036.
2. Vlaeyen, J. W. S.; Maher, C. G.; Wiech, K.; Van Zundert, J.; Meloto, C. B.; Diatchenko, L.; Battie, M. C.; Goossens, M.; Koes, B.; Linton, S. J., Low back pain. *Nat Rev Dis Primers* **2018,** *4* (1), 52.
3. Freemont, A. J., The cellular pathobiology of the degenerate intervertebral disc and discogenic back pain. *Rheumatology* **2009,** *48* (1), 5-10.
4. Hoy, D.; Brooks, P.; Blyth, F.; Buchbinder, R., The Epidemiology of low back pain. *Best practice & research. Clinical rheumatology* **2010,** *24* (6), 769-81.
5. Henschke, N.; Maher, C. G.; Refshauge, K. M.; Herbert, R. D.; Cumming, R. G.; Bleasel, J.; York, J.; Das, A.; McAuley, J. H., Prognosis in patients with recent onset low back pain in Australian primary care: inception cohort study. *Bmj* **2008,** *337*, a171.
6. Sakai, D.; Andersson, G. B., Stem cell therapy for intervertebral disc regeneration: obstacles and solutions. *Nature reviews. Rheumatology* **2015,** *11* (4), 243-56.
7. Vadala, G.; Sowa, G.; Hubert, M.; Gilbertson, L. G.; Denaro, V.; Kang, J. D., Mesenchymal stem cells injection in degenerated intervertebral disc: cell leakage may induce osteophyte formation. *Journal of tissue engineering and regenerative medicine* **2012,** *6* (5), 348-355.
8. Krupkova, O.; Hlavna, M.; Amir Tahmasseb, J.; Zvick, J.; Kunz, D.; Ito, K.; Ferguson, S. J.; Wuertz-Kozak, K., An Inflammatory Nucleus Pulposus Tissue Culture Model to Test Molecular Regenerative Therapies: Validation with Epigallocatechin 3-Gallate. *International journal of molecular sciences* **2016,** *17* (10).
9. Johnson, Z. I.; Schoepflin, Z. R.; Choi, H.; Shapiro, I. M.; Risbud, M. V., DISC IN FLAMES: ROLES OF TNF-alpha AND IL-1 beta IN INTERVERTEBRAL DISC DEGENERATION. *European cells & materials* **2015,** *30*, 104-117.
10. Freemont, A. J.; Watkins, A.; Le Maitre, C.; Baird, P.; Jeziorska, M.; Knight, M. T.; Ross, E. R.; O'Brien, J. P.; Hoyland, J. A., Nerve growth factor expression and innervation of the painful intervertebral disc. *The Journal of pathology* **2002,** *197* (3), 286-92.
11. Luo, X. W.; Liu, K.; Chen, Z.; Zhao, M.; Han, X. W.; Bai, Y. G.; Feng, G., Adenovirus-mediated GDF-5 promotes the extracellular matrix expression in degenerative nucleus pulposus cells. *J Zhejiang Univ Sci B* **2016,** *17* (1), 30-42.
12. Bae, W. C.; Masuda, K., Emerging technologies for molecular therapy for intervertebral disk degeneration. *The Orthopedic clinics of North America* **2011,** *42* (4), 585-601, ix.
13. Le Maitre, C. L.; Freemont, A. J.; Hoyland, J. A., Expression of cartilage-derived morphogenetic protein in human intervertebral discs and its effect on matrix synthesis in degenerate human nucleus pulposus cells. *Arthritis research & therapy* **2009,** *11* (5), R137.
14. Qi, L. S.; Larson, M. H.; Gilbert, L. A.; Doudna, J. A.; Weissman, J. S.; Arkin, A. P.; Lim, W. A., Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **2013,** *152* (5), 1173-83.
15. Kaneti, L.; Bronshtein, T.; Malkah Dayan, N.; Kovregina, I.; Letko Khait, N.; Lupu-Haber, Y.; Fliman, M.; Schoen, B. W.; Kaneti, G.; Machluf, M., Nanoghosts as a Novel Natural Nonviral Gene Delivery Platform Safely Targeting Multiple Cancers. *Nano letters* **2016,** *16* (3), 1574-82.

16. Bourgine, P.; Le Magnen, C.; Pigeot, S.; Geurts, J.; Scherberich, A.; Martin, I., Combination of immortalization and inducible death strategies to generate a human mesenchymal stromal cell line with controlled survival. *Stem Cell Res* **2014,** *12* (2), 584-98.
17. Sultankulov, B.; Berillo, D.; Sultankulova, K.; Tokay, T.; Saparov, A., Progress in the Development of Chitosan-Based Biomaterials for Tissue Engineering and Regenerative Medicine. *Biomolecules* **2019,** *9* (9).
18. Kwang, T. W.; Zeng, X.; Wang, S., Manufacturing of AcMNPV baculovirus vectors to enable gene therapy trials. *Mol Ther Methods Clin Dev* **2016,** *3*, 15050.
19. Ono, C.; Ninomiya, A.; Yamamoto, S.; Abe, T.; Wen, X.; Fukuhara, T.; Sasai, M.; Yamamoto, M.; Saitoh, T.; Satoh, T.; Kawai, T.; Ishii, K. J.; Akira, S.; Okamoto, T.; Matsuura, Y., Innate immune response induced by baculovirus attenuates transgene expression in mammalian cells. *J Virol* **2014,** *88* (4), 2157-67.
20. Facciabene, A.; Aurisicchio, L.; La Monica, N., Baculovirus vectors elicit antigen-specific immune responses in mice. *J Virol* **2004,** *78* (16), 8663-72.
21. Chan, S. C.; Burki, A.; Bonel, H. M.; Benneker, L. M.; Gantenbein-Ritter, B., Papain-induced in vitro disc degeneration model for the study of injectable nucleus pulposus therapy. *The spine journal : official journal of the North American Spine Society* **2013,** *13* (3), 273-83.
22. Gay, M. H.; Mehrkens, A.; Rittmann, M.; Haug, M.; Barbero, A.; Martin, I.; Schaeren, S., Nose to back: compatibility of nasal chondrocytes with environmental conditions mimicking a degenerated intervertebral disc. *European cells & materials* **2019,** *37*, 214-232.
23. Regmi, S.; Pathak, S.; Kim, J. O.; Yong, C. S.; Jeong, J. H., Mesenchymal stem cell therapy for the treatment of inflammatory diseases: Challenges, opportunities, and future perspectives. *Eur J Cell Biol* **2019,** *98* (5-8), 151041.
24. Ousterout, D. G.; Kabadi, A. M.; Thakore, P. I.; Majoros, W. H.; Reddy, T. E.; Gersbach, C. A., Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. *Nature communications* **2015,** *6*.
25. Mayer, J. E.; Iatridis, J. C.; Chan, D.; Qureshi, S. A.; Gottesman, O.; Hecht, A. C., Genetic polymorphisms associated with intervertebral disc degeneration. *Spine Journal* **2013,** *13* (3), 299-317.
26. Krupkova, O.; Cambria, E.; Besse, L.; Besse, A.; Bowles, R.; Wuertz-Kozak, K., The potential of CRISPR/Cas9 genome editing for the study and treatment of intervertebral disc pathologies *JOR Spine* **2018,** *1* (1).
27. Miyamoto, Y.; Mabuchi, A.; Shi, D.; Kubo, T.; Takatori, Y.; Saito, S.; Fujioka, M.; Sudo, A.; Uchida, A.; Yamamoto, S.; Ozaki, K.; Takigawa, M.; Tanaka, T.; Nakamura, Y.; Jiang, Q.; Ikegawa, S., A functional polymorphism in the 5' UTR of GDF5 is associated with susceptibility to osteoarthritis. *Nat Genet* **2007,** *39* (4), 529-33.
28. Mumme, M.; Steinitz, A.; Nuss, K. M.; Klein, K.; Feliciano, S.; Kronen, P.; Jakob, M.; von Rechenberg, B.; Martin, I.; Barbero, A.; Pelttari, K., Regenerative Potential of Tissue-Engineered Nasal Chondrocytes in Goat Articular Cartilage Defects. *Tissue engineering. Part A* **2016,** *22* (21-22), 1286-1295.
29. Mumme, M.; Barbero, A.; Miot, S.; Wixmerten, A.; Feliciano, S.; Wolf, F.; Asnaghi, A. M.; Baumhoer, D.; Bieri, O.; Kretzschmar, M.; Pagenstert, G.; Haug, M.; Schaefer, D. J.; Martin, I.; Jakob, M., Nasal chondrocyte-based engineered autologous cartilage tissue for repair of articular cartilage defects: an observational first-in-human trial. *Lancet* **2016,** *388* (10055), 1985-1994.
30. Pelttari, K.; Pippenger, B.; Mumme, M.; Feliciano, S.; Scotti, C.; Mainil-Varlet, P.; Procino, A.; von Rechenberg, B.; Schwamborn, T.; Jakob, M.; Cillo, C.; Barbero, A.; Martin, I., Adult human neural crest-derived cells for articular cartilage repair. *Science translational medicine* **2014,** *6* (251), 251ra119.
31. Murphy, D. E.; de Jong, O. G.; Brouwer, M.; Wood, M. J.; Lavieu, G.; Schiffelers, R. M.; Vader, P., Extracellular vesicle-based therapeutics: natural versus engineered targeting and trafficking. *Exp Mol Med* **2019,** *51* (3), 1-12.
32. Tan, S.; Wu, T.; Zhang, D.; Zhang, Z., Cell or cell membrane-based drug delivery systems. *Theranostics* **2015,** *5* (8), 863-81.
33. Lupu-Haber, Y.; Bronshtein, T.; Shalom-Luxenburg, H.; D'Atri, D.; Oieni, J.; Kaneti, L.; Shagan, A.; Hamias, S.; Amram, L.; Kaneti, G.; Cohen Anavy, N.; Machluf, M., Pretreating Mesenchymal Stem Cells with Cancer Conditioned-Media or Proinflammatory Cytokines Changes the Tumor and Immune Targeting by Nanoghosts Derived from these Cells. *Advanced healthcare materials* **2019,** *8* (10), e1801589.