Stimulation of the reparative activities in human chondral defects upon rAAV-mediated overexpression of *sox9* and TGF-β via vector delivery in an alginate-based hydrogel

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INTRODUCTION: Recombinant adeno-associated virus (rAAV) emerged as promising gene delivery vectors for interventions in human traumatic articular cartilage defects and osteoarthritic lesions through the transfer of specific therapeutic gene sequences [1]. Despite this potential, *in vivo* application of gene therapies remains challenging especially to achieve an adapted and targeted expression of the transgenes within optimal temporal and spatial parameters. The present study aimed at exploring the ability of reparative rAAV vectors carrying *sox9* and TGF-β sequences provided via an alginate scaffold to trigger biological and reparative cell activities in human chondral defects *in situ* as workable, off-the-shelf platforms for future applications in sites of articular cartilage damage.

METHODS: rAAV vectors were packaged, purified, and titrated as previously described [2-5]. rAAV-*lacZ* carries the *E. coli* β-galactosidase (*lacZ*) reporter gene, rAAV-FLAG-h*sox9* a 1.7 kb human FLAG-tagged *sox9* sequence, and rAAV-hTGF-β a 1.2-kb human transforming growth factor beta 1 sequence, all controlled by the CMV-IE promoter/enhancer [2-5]. Human osteoarthritic articular cartilage biopsies (n = 9; 6-mm diameter; Mankin score = 7-9) randomly collected from the femoral condyle of patients undergoing total knee arthroplasty were used to create standardized chondral defects with a 2-mm biopsy punch and incubated at 37ºC for 2-3 days before addition of the various hydrogel systems for up to 21 days [3,4]. rAAV vectors (10 µl per defect) were formulated in 3% alginate (AlgPH155, Danisco) hydrogel (final alginate concentration 0.2%) by direct mixing in a syringe [5]. The defects were collected, fixed, and subsequently decalcified, embedded, sectioned, and prepared for the following analyses [2-5]. Transgene (*sox9*, TGF-β) expression was detected by immunocytochemistry [2-4]. Toluidine blue staining was performed on fixed tissues for evaluating the deposition of cartilage matrix proteoglycans [2-4]. Immunohistochemical analyses were performed to monitor the deposition of type-II/-I/-X collagen [2-4]. The immunostained and stained sections were scored for uniformity and intensity according to modified Bern score grading system as: 0 (no staining), 1 (heterogenous and/or weak staining), 2 (homogeneous and/or moderate staining), 3 (homogeneous and/or intense staining) and 4 (very intense staining) [4]. Each condition was performed in duplicate in three independent experiments. The Shapiro-Wilk test and Kruskal-Wallis test were employed with $P < 0.05$ considered statistically significant.

RESULTS: Successful, significant therapeutic rAAV gene delivery and overexpression via alginate hydrogel delivery was achieved in the defects over time (21 days) as noted by the effective immunodetection of SOX9 and TGF-β (**Figure 1a**) when providing systems formulating rAAV-FLAG-h*sox9* and rAAV-hTGF-β, respectively, relative to rAAV-*lacZ* and no vector treatments (3.5- and 2-fold difference with rAAV-FLAG-h*sox9* and rAAV-hTGF-β, respectively, relative to rAAV-*lacZ*, always $P < 0.05$, and 3.5- and 1.5-fold difference, respectively, relative to the no vector treatment, $P < 0.05$ and $P = 0.0546$, respectively) (**Figures 1d and 1g**). Specific rAAV-mediated *sox9* and TGF-β overexpression (rAAV-FLAG-h*sox9* and rAAV-hTGF-β, respectively) via alginate hydrogel delivery significantly increased the cell reparative activities in the defects over time (21 days) relative to rAAV-*lacZ* and no vector treatment, as seen by an increased deposition of cartilage matrix proteoglycans (always 1.3-fold difference relative to rAAV-*lacZ* and to the no vector treatment, always $P < 0.05$) and of type-II collagen (2.6- and 1.6-fold difference, respectively, relative to rAAV-*lacZ*, $P < 0.05$ and $P = 0.0625$, and 2- and 1.4-fold difference, respectively, relative to the no vector treatment, $P = 0.0576$ and $P > 0.99$) (**Figures 1b, 1e, and 1h**). Interestingly, specific rAAV-mediated *sox9* and TGF-β overexpression (rAAV-FLAG-h*sox9* and rAAV-hTGF-β, respectively) via alginate hydrogel delivery advantageously prevented premature osteogenic and hypertrophic commitment as seen by a decreased deposition of type-I collagen (2- and 2-fold difference, respectively, relative to rAAV-*lacZ*, always $P = 0.1417$) and of type-X collagen (3- and 3-fold difference, respectively, relative to rAAV-*lacZ*, always $P < 0.05$, and always 1.5-fold difference relative to the no vector treatment, always $P = 0.6206$) (**Figures 1c, 1f, and 1i**).

DISCUSSION: The current study reveals the potential of using alginate hydrogel-based controlled delivery of rAAV *sox9* and TGF-β vectors as a workable approach to trigger the reparative properties of chondral cartilage defects for future chondrotherapeutic applications.

SIGNIFICANCE/CLINICAL RELEVANCE: This evaluation reports a strong tool to provide therapeutic rAAV vectors via an alginate hydrogel as a means to directly heal injured human articular cartilage.

REFERENCES: [1] Cucchiarini & Madry, *Nat Rev Rheumatol*. 2019, 15:18; [2] Venkatesan *et al*., *J Transl Med*. 2013, 11:211; [3] Rey-Rico *et al*., *Int J Nanomedicine*. 2017, 12:6985; [4] Morscheid *et al*., *J Clin Med.* 2019, 28:1326; [5] Díaz-Rodríguez *et al*., *Int J Pharm.* 2015, 496:614.

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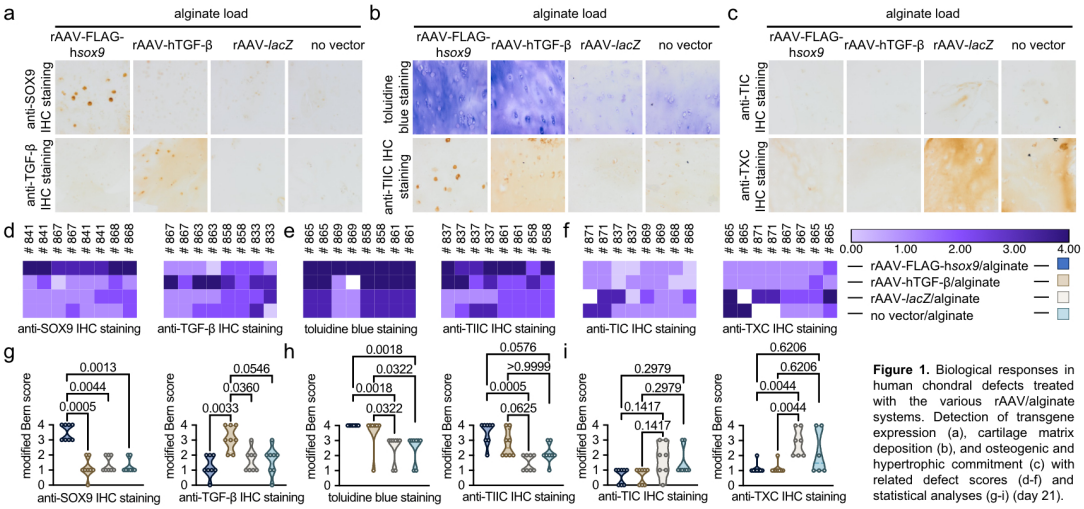

Figure 1. Biological responses in human chondral defects treated with the various rAAV/alginate systems. Detection of transgene expression (a), cartilage matrix deposition (b), and osteogenic and hypertrophic commitment (c) with related defect scores (d-f) and statistical analyses (g-i) (day 21).