Enhancement of Chondrogenic Differentiation in Human Mesenchymal Stromal Cells Via Carbon Dot-Facilitated rAAV-Mediated Delivery of *sox9* and TGF-β

Jagadeesh K. Venkatesan[1]; Gertrud Schmitt[1]; Fanfan Li[1]; Henning Madry[1]; Françoise Pons[2]; Luc Lebeau[2]; Magali Cucchiarini[1]
[1]Saarland University Medical Center, Homburg/Saar, Germany
[2]University of Strasbourg, UMR 7199, Faculté de Pharmacie, Illkirch, France

INTRODUCTION:

The articular cartilage is essential for joint support and movement, yet it exhibits a limited capacity for self-repair after injury. Recombinant adeno-associated virus (rAAV) vectors offer a promising avenue for the long-term treatment of articular cartilage defects [1]. However, the efficacy of these vectors is often compromised by the presence of neutralizing antibodies against AAV capsid components in a high number of patients [2]. Carbon dots, an emerging class of nanocarriers, have been reported for their ability to deliver DNA sequences in a variety of cell lines [3], possibly providing novel platforms to safely deliver rAAV vectors [4]. Here, we assessed the potential of a smart carbon dot (CD-2) compound to facilitate the delivery of chondrogenic rAAV *sox9* and TGF-β vectors in human bone marrow-derived mesenchymal stromal cells (hBMMSCs), a viable source of reparative, transplantable cells capable of enhancing cartilage repair [5].

METHODS:

rAAV vectors were packaged, purified, and titrated as previously described [6,7]. rAAV-*lacZ* carries the *E. coli* β-galactosidase (*lacZ*) reporter gene, rAAV-FLAG-h*sox9* carries a human *sox9* sequence, and rAAV-hTGF-β1 a human transforming growth factor beta 1, all controlled by the CMV-IE promoter/enhancer [6,7]. Bone marrow aspirates were obtained from the distal femurs of donors undergoing total knee arthroplasty (n = 2). hBMMSCs were isolated [6], seeded in 48-well plates (5,000 cells at passage 1/well), and maintained in DMEM, 10% FBS, 100 U/ml penicillin G, and 100 µl/ml streptomycin at 37ºC during 12 h before the experiments. The CD-2 compound was generated through pyrolysis of citric acid in the presence of various additives (pentaethylenehexamine - PEHA, *N,N*-dimethylethylenediamine - DMEDA, branched poly(ethyleneimine) 600 Da - bPEI600, poly(ethylene glycol) methyl ether 550 Da - mPEG550, 2 kDa mPEG2000) [3], mixed with rAAV-FLAG-h*sox9*, rAAV-hTGF-β1, or rAAV-*lacZ* in equal parts (v:v), and applied to high-density aggregate cultures of hBMMSCs (2 x 10^5 cells) in chondrogenic medium (DMEM high glucose, ITS$^+$ Premix, 1 mM pyruvate, 37.5 µg/ml ascorbate 2-phosphate, 10^{-7} M dexamethasone, 10 ng/ml TGF-β) for up to 21 days. CD-2 lacking rAAV was used as a control treatment. Transgene expression was analyzed by immunohistochemistry on fixed cultures (SOX9, TGF-β1) and by ELISA (TGF-β1) (R&D Systems) [6,7]. The deposition of type-II, -I, and -X collagen was monitored by immunohistochemistry on fixed cultures [6,7]. The proteoglycan contents were monitored by binding to the dimethylmethylene blue dye and the DNA contents by Hoechst 33258 assay [6,7]. Total RNA was extracted using the RNeasy Protect Mini kit (Qiagen) and reverse transcription was carried out with the 1st Strand cDNA Synthesis kit (Qiagen). cDNA amplification was performed via SYBR Green real-time RT-PCR [6]. Ct values were obtained for each target gene (SOX9, type-II collagen - COL2A1, type-I collagen - COL1A1, type-X collagen - COL10A1, aggrecan - ACN) and for GAPDH as a control for normalization. Fold inductions (relative to control samples) were measured using the $2^{-\Delta\Delta Ct}$ method [6]. Each condition was performed in duplicate in three independent experiments. The t-test was employed with $P < 0.05$ considered statistically significant.

RESULTS:

Delivery of the rAAV *sox9* and TGF-β vectors via the CD-2 compound in aggregate cultures of hBMMSCs allowed to promote an effective overexpression of each transgene after 21 days as seen by respective, intense signals of SOX9 and TGF-β immunoreactivity compared with the control conditions (delivery of rAAV-*lacZ*, CD-2 lacking rAAV) (**Fig. 1A**), a finding validated for TGF-β by ELISA (**Fig. 1B**). Effective chondrogenic differentiation of hBMMSCs in the aggregate cultures was noted after 21 days when applying the rAAV *sox9* and TGF-β vectors via the CD-2 compound relative to the control conditions as seen by higher type-II collagen deposition (**Fig. 2**), as corroborated by real-time RT-PCR analysis (up to 2.3- and 1.9-fold increases in COL2A1 expression with rAAV *sox9* and TGF-β, respectively, compared with the control conditions, $P \leq 0.001$) (**Fig. 3**), probably due to respective increases in SOX9 expression (**Fig. 3**). Interestingly, a real-time RT-PCR analysis further revealed increases in ACAN expression with rAAV *sox9* and TGF-β using CD-2 in the hBMMSC aggregate cultures after 21 days (up to 1.7- and 1.6-fold increases, respectively, compared with the control conditions, $P \leq 0.001$) (**Fig. 3**). In contrast, administration of the rAAV *sox9* and TGF-β vectors via the CD-2 compound in the hBMMSC aggregate cultures after 21 days decreased the deposition of type-I and -X collagen (especially *sox9*) relative to the control conditions (**Fig. 2**), a finding supported by a real-time RT-PCR analysis (COL1A1: up to 1.4- and 1.7-fold decreases, respectively, compared with the control conditions, $P \leq 0.001$; COL10A1: up to 3.9- and 1.2-fold decreases, respectively, compared with the control conditions, $P \leq 0.001$) (**Fig. 3**). Of further note, rAAV-mediated overexpression of *sox9* and TGF-β enhanced the proteoglycan contents in the hBMMSC aggregate cultures after 21 days compared with the control conditions (1.1- and 1.3-fold, respectively, $P \leq 0.001$) (**Table 1**).

DISCUSSION:

The application of rAAV *sox9* and TGF-β via the CD-2 compound enhances the chondrogenic differentiation processes in aggregate cultures of hBMMSCs.

SIGNIFICANCE:

These findings highlight the potential of CD-2 as a novel compound to deliver therapeutic rAAV vectors for the future treatment of articular cartilage defects.

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ACKNOWLEDGMENTS:

Supported by a grant from German Research Society (*Deutsche Forschungsgemeinschaft*, DFG VE 1099/3-1 to JKV).

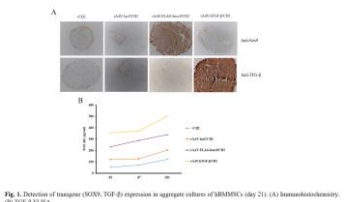

Fig. 1. Detection of transgene (SOX9, TGF-β) expression in aggregate cultures of hBMMSCs (day 21). (A) Immunohistochemistry. (B) TGF-β ELISA.

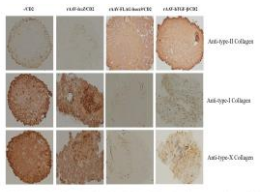

Fig. 2. Immunohistochemical detection of the deposition of type-II, -I, and -X collagen in aggregate cultures of hBMMSCs (day 21).

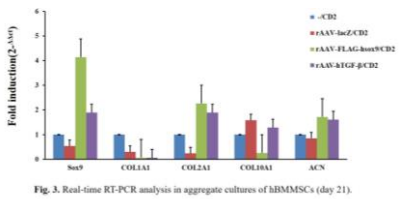

Fig. 3. Real-time RT-PCR analysis in aggregate cultures of hBMMSCs (day 21).

Table 1. Biochemical analysis in aggregate cultures of hBMMSCs (day 21)

Assay	-/CD2	rAAV-*lacZ*/CD2	rAAV-FLAG-h*sox9*/CD2	rAAV-hTGF-β/CD2
PGs/total proteins (ug/ug)	0.98 (0.1)	0.99 (0.14)	1.03 (0.02)*	1.31 (0.08)*
DNA/total proteins (ng/ug)	0.4 (0.03)	0.4 (0.10)	0.3 (0.02)	0.4 (0.01)
PGs/DNA (ug/ng)	2.5 (0.23)	2.4 (0.27)	3.4 (0.26)*	3.2 (0.12)*

PGs: proteoglycans. *Statistically significant compared with the control conditions.