

Overexpression of human DKK1 via rAAV vector-mediated gene transfer stimulates chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells

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Abstract

Enhancing the chondroregenerative activities of mesenchymal stem cells via therapeutic gene transfer as reinforced sources of implantable cells in sites of cartilage injury is a promising tool to improve the natural processes of cartilage repair. In the present study, we show that overexpression of the Dickkopf-related protein 1 (DKK1) via clinically adapted recombinant adeno-associated viral (rAAV) vectors is capable of significantly stimulating proliferative, anabolic, and chondrodifferentiation events in primary human mes-

enchymal stem cells compared with control (reporter rAAV *lacZ*) transduction over an extended period of time *in vitro* (21 days). Strikingly, administration of the rAAV DKK1 candidate vector concomitantly restrained unwanted osteogenic and hypertrophic differentiation outcomes in these cells. These findings reveal the possible future benefits of such an approach to treat articular cartilage lesions in relevant experimental models *in vivo*.

Introduction

In absence of vascularization and of potential delivery of chondroreparative progenitor cells, the adult articular cartilage is not capable of fully recovering from injury (trauma, degenerative osteoarthritis), leading to a repair tissue of fibrocartilaginous nature (type-I collagen) with poor mechanical properties relative to the original hyaline cartilage (type-II collagen, proteoglycans) (Frisbie *et al.* 2003, Madry *et al.* 2010) even after surgical interventions like marrow stimulation procedures (Madry *et al.* 2010). Repopulation of cartilage lesions by administration of mesenchymal stem cells (MSCs) such as those originating from the bone marrow compartment is a promising approach to enhance the repair of damaged articular cartilage (Johnstone *et al.* 2013). Yet, all attempts at implanting MSCs in patients met limited success, with outcomes associated again with the formation of fibrocartilage in sites of injury showing poor integration with the surrounding unaffected cartilage (Skowronski & Rutka 2013, Wakitani *et al.* 2004).

Genetic modification of MSCs prior to re-implantation in cartilage defects represents a promising approach to enhance the chondroreparative activities of this highly specialized tissue (Baksh *et al.* 2004, Cucchiarini & Madry 2010, Evans *et al.* 2000, Yoo *et al.* 2000). A number of sequences were reported for their chondroreparative activities among which genes for ECM components (cartilage oligomeric matrix protein - COMP) (Haleem-Smith *et al.* 2012), growth factors and cytokines (transforming growth factor beta - TGF- β ; bone morphogenetic proteins - BMPs; basic fibroblast growth factor - FGF-2; insulin-like growth factor I - IGF-I; interleukin 10 - IL-10) (Cucchiarini *et al.* 2011, Frisch *et al.* 2014a, 2014b, Liu *et al.* 2015, Pagnotto *et al.* 2007, Steinert *et al.* 2009, 2012), transcription factors (SOX family, zinc finger protein 145 - ZNF145) (Ikeda *et al.* 2004, Kim & Im 2011, Liu *et al.* 2011, Venkatesan *et al.* 2012), signaling molecules (Indian hedgehog - Ihh, Wnt11) (Liu *et al.* 2014, Steinert *et al.* 2012), and other therapeutic compounds (small interfering RNA - siRNA - against the osteogenic Cbfa-1 transcription factor) (Jeon *et al.*

2012). However, thus far none of these candidates proved reliably effective to treat sites of cartilage injury *in vivo* (Cucchiariini & Madry 2005), showing the critical need to identify new targets for therapy.

The goal of the present study was to evidence potential metabolic and chondrogenic effects of the Dickkopf-related protein 1 (DKK1), an inhibitor of the canonical wingless-type (Wnt) signaling pathway involved in patterning, joint development, and progenitor cell differentiation in osteoblasts (Fedi *et al.* 1999, Glinka *et al.* 1998, Sandell & Adler 1999), in light of the protective effects of this factor against cartilage destruction and osteophyte formation in experimental models of osteoarthritis (Funck-Brentano *et al.* 2014, Oh *et al.* 2012). To achieve this goal, a sequence coding for DKK1 was delivered to human MSCs (hMSCs) via recombinant adeno-associated viral (rAAV) vectors as these constructs are known to transduce such cells at elevated efficiencies (up to 100%) for prolonged periods of time (at least 3 weeks *in vitro*) and without impeding their potential for differentiation (Cucchiariini *et al.* 2011, Pagnotto *et al.* 2007, Venkatesan *et al.* 2012), making rAAV the currently best suited systems for translational orthopaedic applications (Evans & Huard 2015, Madry & Cucchiariini 2016).

The present work demonstrates that effective, prolonged overexpression of DKK1 can be achieved in hMSCs via rAAV-mediated gene transfer, leading to enhanced levels of cell proliferation, ECM deposition, and chondrogenic differentiation in the cells relative to control treatments and to the prevention of undesirable osteogenic/hypertrophic differentiation *in vitro*. These findings provide a basis to further test the current rAAV DKK1 construct as a promising tool to treat articular cartilage defects *in vivo*.

Materials and Methods

Reagents

All reagents were from Sigma (Munich, Germany) unless otherwise indicated. Recombinant FGF-2 was purchased at Peprotech (Hamburg, Germany). Recombinant TGF- β 3 was from R&D Systems (Wiesbaden, Germany). Dimethylmethyle blue was from Serva (Heidelberg, Germany). The anti-DKK1 antibody (B-7) was from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany), the anti-type-II collagen (II-II6B3) antibody from the NIH Hybridoma Bank (University of Iowa, Ames, USA), the anti-type-I collagen (COL-1) antibody from Abcam (Cambridge, UK), and the anti-type-X collagen (COL-10) antibody from Sigma. Secondary biotinylated secondary antibodies and the ABC kit were from Vector Laboratories

(Grünberg, Germany). The DKK1 enzyme-linked immunosorbent assay (Quantikine Human Dkk-1 ELISA) was from R&D Systems and the type-II collagen ELISA (Arthrogen-CIA Capture ELISA kit) from Chondrex (Redmond, WA, USA).

Cell culture

Bone marrow aspirates (~ 10 ml) were obtained from the distal femurs of donors undergoing total knee arthroplasty (n = 8) (age 75 ± 3 years). The study was approved by the Ethics Committee of the Saarland Physicians Council (Ha06/08). All patients provided informed consent to participate in the study before inclusion in the study. All procedures were in accordance with the Helsinki Declaration. All patients provided informed consent to report individual patient data. hMSCs were isolated and expanded in culture according to standard protocols (Venkatesan *et al.* 2012). Briefly, aspirates were washed in Dulbecco's modified Eagle's medium (DMEM) and centrifuged, and the pellet was resuspended in Red Blood Cell Lysing Buffer in DMEM (1:1). The resulting fraction was washed, pelleted, and resuspended in DMEM containing 10% fetal bovine serum with 100 U/ml penicillin and 100 μ l/ml streptomycin (growth medium). Cells were plated in T75 flasks and maintained at 37°C in a humidified atmosphere with 5% CO₂. The medium was exchanged after 24 h and every 2-3 days thereafter using growth medium with recombinant FGF-2 (1 ng/ml). Cells were detached and replated for further experiments at the appropriate densities. hMSCs were analyzed by flow cytometry for expression of stem cell surface markers (CD71⁺, CD105⁺, CD34⁻) (Venkatesan *et al.* 2012). All experiments were performed with cells at no more than passage 2.

Plasmids and rAAV vectors

The constructs were derived from pSSV9, an AAV-2 genomic clone (Samulski *et al.* 1987, 1989). rAAV-*lacZ* carries the *lacZ* gene for *E. coli* β -galactosidase (β -gal) (Cucchiariini *et al.* 2003, Venkatesan *et al.* 2012). A human DKK1 sequence (0.8 kb) (Origene) was cloned in place of the *lacZ* gene in rAAV-*lacZ* (Cucchiariini *et al.* 2003). The resulting plasmid vector is rAAV-hDKK1 where the presence of the DKK1 fragment was confirmed by sequencing. In both vectors, transgene expression is placed under the control of the cytomegalovirus immediate-early (CMV-IE) promoter (Cucchiariini *et al.* 2003, Venkatesan *et al.* 2012). The vectors were packaged as conventional (not self-complementary) vectors using a helper-free, two-plasmid transfection system in the 293 packaging cell line (an adenovirus-transformed human embryonic kidney cell line) with the packaging plasmid pXX2

and the Adenovirus helper plasmid pXX6 as previously described (Venkatesan *et al.* 2012). The vector preparations were purified by dialysis and titered by real-time PCR (Cucchiaroni *et al.* 2003, Venkatesan *et al.* 2012), averaging 10^{10} transgene copies/ml (viral particles to functional vectors = 500/1).

rAAV-mediated gene transfer

Cells were transduced with rAAV vectors in monolayer culture (2×10^4 cells, 20 μ l vector, i.e. 4×10^5 functional recombinant viral particles and MOI = 20) and kept in growth medium for up to 21 days (Venkatesan *et al.* 2012). hMSC aggregate cultures (2×10^5 cells) were prepared and kept in defined chondrogenic medium (high-glucose DMEM 4.5 g/l, penicillin/streptomycin, 6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, 6.25 μ g/ml selenous acid, 5.35 μ g/ml linoleic acid, 1.25 μ g/ml bovine serum albumin, 1 mM sodium pyruvate, 37.5 mg/ml ascorbate 2-phosphate, 10^{-7} M dexamethasone, 10 ng/ml TGF- β 3) for transduction with rAAV (40 μ l vector, i.e. 8×10^5 functional recombinant viral particles, i.e. MOI = 4) for up to 21 days (Venkatesan *et al.* 2012).

Transgene expression

Transgene (DKK1) expression was assayed by ELISA (Cucchiaroni *et al.* 2011, Venkatesan *et al.* 2012). Briefly, samples (monolayer and aggregate cultures) were washed twice and maintained for 24 h in serum-free medium prior to collection of the supernatants of culture at the denoted time points for centrifugation to separate debris. Measurements were carried out on a GENios spectrophotometer/fluorometer (Tecan, Crailsheim, Germany). DKK1 expression was also monitored by immunocyto-/histochemistry on fixed monolayer cultures and on paraffin-embedded sections of aggregates (5 μ m), respectively, using a specific primary antibody (1:100), a biotinylated secondary antibody, and the ABC method with diaminobenzidine (DAB) as the chromogen (Venkatesan *et al.* 2012). Samples were examined by light microscopy (BX 45; Olympus, Hamburg, Germany) (Venkatesan *et al.* 2012). To control for secondary immunoglobulins, samples were processed with omission of the primary antibody.

Biochemical assays

Cultures were harvested and digested with papain (Venkatesan *et al.* 2012). The DNA and proteoglycan contents were determined with a fluorimetric assay using Hoechst 22358 and by binding to dimethylmethylene blue dye, respectively (Venkatesan *et al.* 2012). The type-II collagen contents were monitored by ELISA (Venkatesan *et al.* 2012). Data were nor-

malized to total cellular proteins using a protein assay (Pierce Thermo Scientific, Fisher Scientific, Schwerte, Germany). All measurements were performed with a GENios spectrophotometer/fluorometer.

Histological, immunocytochemical, and immunohistochemical analyses

Cultures were harvested and fixed in 4% formalin, dehydrated in graded alcohols, embedded in paraffin, and sectioned (3 μ m). Sections were stained with toluidine blue (matrix proteoglycans) and alizarin red (matrix mineralization) (Venkatesan *et al.* 2012). Expression of type-II and -X collagen was detected by immunohistochemistry using specific primary antibodies, biotinylated secondary antibodies, and the ABC method with DAB (Venkatesan *et al.* 2012). To control for secondary immunoglobulins, sections were processed with omission of the primary antibody. Samples were examined under light microscopy (BX 45; Olympus).

Histomorphometry

The evaluations were performed at four random standardized sites in monolayer cultures or using ten serial histological and immunohistochemical sections of aggregate cultures for each parameter, test, and replicate condition with the SIS analySIS program (Olympus), Adobe Photoshop (Adobe Systems, Unterschleissheim, Germany), and Scion Image (Scion Corporation, Frederick, MD, USA) (Venkatesan *et al.* 2012). The transduction efficiencies were calculated as the % of X-Gal-stained cells to the total numbers of cells (Venkatesan *et al.* 2017). Toluidine blue and alizarin red staining and type-II and -I collagen immunostaining were scored for uniformity and intensity according to a modified Bern Score grading system (Venkatesan *et al.* 2017) as: 0 (no staining), 1 (heterogeneous and/or weak staining), 2 (homogeneous and/or moderate staining), 3 (homogeneous and/or intense staining), and 4 (very intense staining). Type-X collagen expression was monitored by estimating the percentage of positively stained cells to the total numbers of cells on immunohistochemical sections. All sections were scored blind by two individuals with regard to the conditions.

Real-time PCR analysis

Total cellular RNA was extracted from the cultures using the RNeasy Protect Mini Kit with an on-column RNase-free DNase treatment (Qiagen, Hilden, Germany). RNA was eluted in 30 μ l RNase-free water. Reverse transcription was carried out with 8 μ l eluate using the 1st Strand cDNA Synthesis kit for RT-PCR (AMV; Roche Applied Science) (Venkatesan *et al.* 2012). An aliquot of the cDNA product (2 μ l) was am-

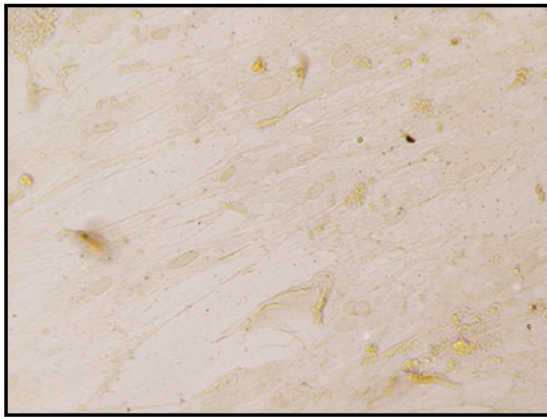
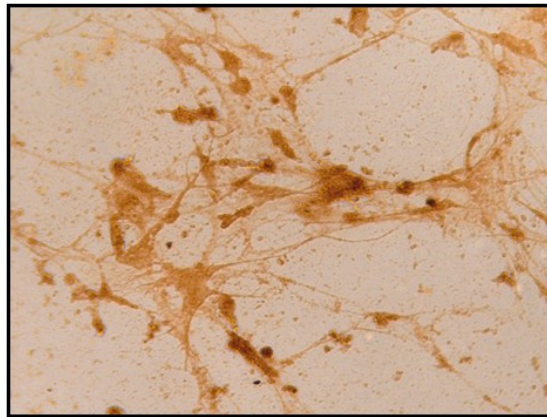
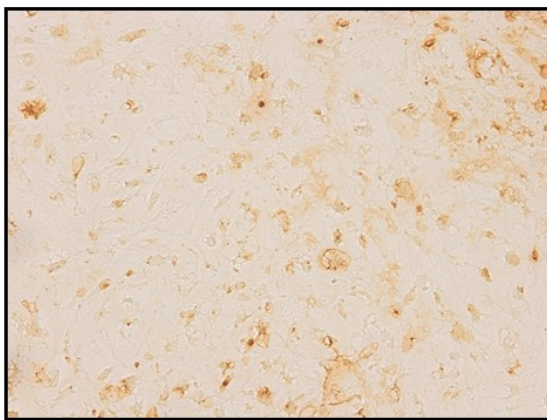
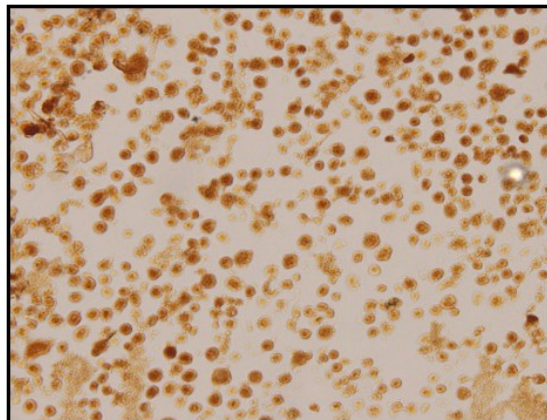
A.**rAAV-lacZ****rAAV-hDKK1****B.****rAAV-lacZ****rAAV-hDKK1**

Figure 1. Detection of transgene (DKK1) expression in rAAV-transduced hMSCs. Cells in monolayer cultures ($n = 24$ using 8 bone marrow aspirates in three independent experiments) (**A**) and aggregate cultures ($n = 24$ using 8 bone marrow aspirates in three independent experiments) (**B**) were transduced in triplicate with rAAV-hDKK1 ($n = 72$ for each type of culture) or rAAV-lacZ ($n = 72$ for each type of culture) (**A**: 20 μ l each vector, MOI = 20; **B**: 40 μ l each vector, MOI = 4) and all the cultures and conditions were processed after 21 days to detect DKK1 expression by specific immunocyto-/histochemistry as described in the Materials and Methods (magnification x20; all representative data).

plified with real-time PCR using the Brilliant SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Waldbronn, Germany) on an Mx3000P QPCR operator system (Stratagene) as follows: initial incubation (95°C, 10 min), amplification for 55 cycles (denaturation at 95°C, 30 sec; annealing at 55°C, 1 min; extension at 72°C, 30 sec), denaturation (95°C, 1 min), and final incubation (55°C, 30 sec) (Venkatesan *et al.* 2012). The primers (Invitrogen, Darmstadt, Germany) used were SOX9 (chondrogenic marker) (forward, 5'-ACACACAGCTCACTCGACCTTG-3'; reverse, 5'-GGGAATTCTGGTTGGTCCTCT-3'), type-II collagen (COL2A1; chondrogenic marker) (forward, 5'-GGACTTTTCTCCCTCTCT-3'; reverse,

5'-GACCCGAAGGTCTTACAGGA-3'), type-I collagen (COL1A1; osteogenic marker) (forward 5'-ACGTCCTGGTGAAG TTGGTC-3'; reverse 5'-ACCAGGGAAGCCTCTCTCTC- 3'), type-X collagen (COL10A1; marker of hypertrophy) (forward 5'-CCCTCTTGTTAG TGCCAACC-3'; reverse 5'-AGATTCCAGTCCTTGGGTCA-3'), runt-related transcription factor 2 (RUNX2; osteogenic marker) (forward 5'-GCAGTTCCCAAGCATTTCAT-3'; reverse 5'-CACTCTGGCTTTGGAAAGAG-3'), β -catenin (mediator of the Wnt signaling pathway for osteoblast lineage differentiation) (forward 5'-CAAGTGGGTGGTATAGAGG-3'; reverse 5'-GCGGGACAAAGGGCAAGA-3'), and glyceralde-

Table 1. DKK1 expression in rAAV-transduced undifferentiated hMSC monolayer cultures and in chondrogenically-induced hMSC aggregate cultures.

System	rAAV- <i>lacZ</i>			rAAV-hDKK1		
	Day 3	Day 7	Day 21	Day 3	Day 7	Day 21
Monolayer cultures	0.05 (0.01)	0.02 (0.01)	0.01 (0.01)	0.60 (0.03)*	0.42 (0.04)*	0.36 (0.03)*
Aggregate cultures	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.18 (0.02)*	0.14 (0.02)*	0.11 (0.01)*

hyde-3-phosphate dehydrogenase (housekeeping gene and internal control) (forward, 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse, 5'-GAAGATGGTGATGGGATTTC-3') (all 150 nM final concentration) (Venkatesan *et al.* 2012). Control conditions included reactions using water and nonreverse-transcribed mRNA. Specificity of the products was confirmed by melting curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) value for each gene of interest was measured for each amplified sample using MxPro QPCR software (Stratagene), and values were normalized to glyceraldehyde-3-phosphate dehydrogenase expression by using the $2^{-\Delta\Delta C_t}$ method, as described previously (Venkatesan *et al.* 2012).

Statistical analysis

All bone marrow aspirates ($n = 8$) were used and tested independently for all the tests performed here (ELISA; biochemical, histological, immunocytochemical, and immunohistochemical analyses; histomorphometry; real-time reverse transcription polymerase chain reaction analyses) and for each replicate condition. Each condition (undifferentiated monolayer cell cultures and chondrogenically-induced aggregate cultures; cells transduced with rAAV-hDKK1 *versus* rAAV-*lacZ* with respective MOIs; variable time points) was performed in triplicate in three independent experiments. Three monolayer cultures were analyzed at four random standardized sites for each test and replicate condition. Ten serial histological and immunohistochemical sections of aggregate cultures were analyzed for each test and replicate condition. Data are expressed as mean \pm standard deviation (SD) of separate experiments. Statistical analysis was

performed using SPSS version 23 (IBM, Armonk, NY, USA) and the t-test was employed to compare related samples to evaluate differences between groups. *P* values of less than 0.05 were considered statistically significant.

Results

DKK1 overexpression in human bone marrow-derived mesenchymal stem cells

Human bone marrow-derived mesenchymal stem cells (hMSCs) were first transduced with rAAV-hDKK1 in undifferentiated monolayer cultures to evaluate the feasibility of overexpressing this factor in these cells relative to the control rAAV-*lacZ* condition. Highly effective, sustained DKK1 expression was significantly achieved in rAAV-hDKK1-transduced hMSCs over a prolonged period of time (21 days), reaching $97 \pm 3\%$ transduction efficiencies *versus* $12 \pm 3\%$ in the presence of rAAV-*lacZ* (8.1-fold difference, $P \leq 0.001$) (Figure 1A). An estimation of the DKK1 production levels by ELISA in the samples revealed significantly higher amounts of factor synthesis with rAAV-hDKK1 relative to rAAV-*lacZ* at any time point of the analysis (up to 36-fold difference, $P \leq 0.001$) (Table 1).

hMSCs were next transduced with rAAV-hDKK1 in chondrogenically-induced aggregate cultures to further evidence the levels of DKK1 expression in such a specific environment compared with control rAAV-*lacZ* treatment. Concordant with the findings in monolayer culture, a strong, significant signal was again noted in the DKK1-treated aggregates

Table 2. Biochemical analyses in rAAV-transduced chondrogenically-induced hMSC aggregate cultures (day 21).

Assay	rAAV- <i>lacZ</i>	rAAV-hDKK1
DNA (ng/mg total proteins)	0.16 (0.02)	0.19 (0.03)*
Proteoglycans (μ g/mg total proteins)	2.2 (0.4)	3.4 (0.6)*
Type-II collagen (ng/mg total proteins)	66.1 (12.2)	101.4 (17.3)*
Proteoglycans/DNA (μ g/ng)	13.8 (1.2)	17.9 (1.8)*
Type-II collagen/DNA (ng/ng)	412.5 (20.3)	531.6 (28.4)*

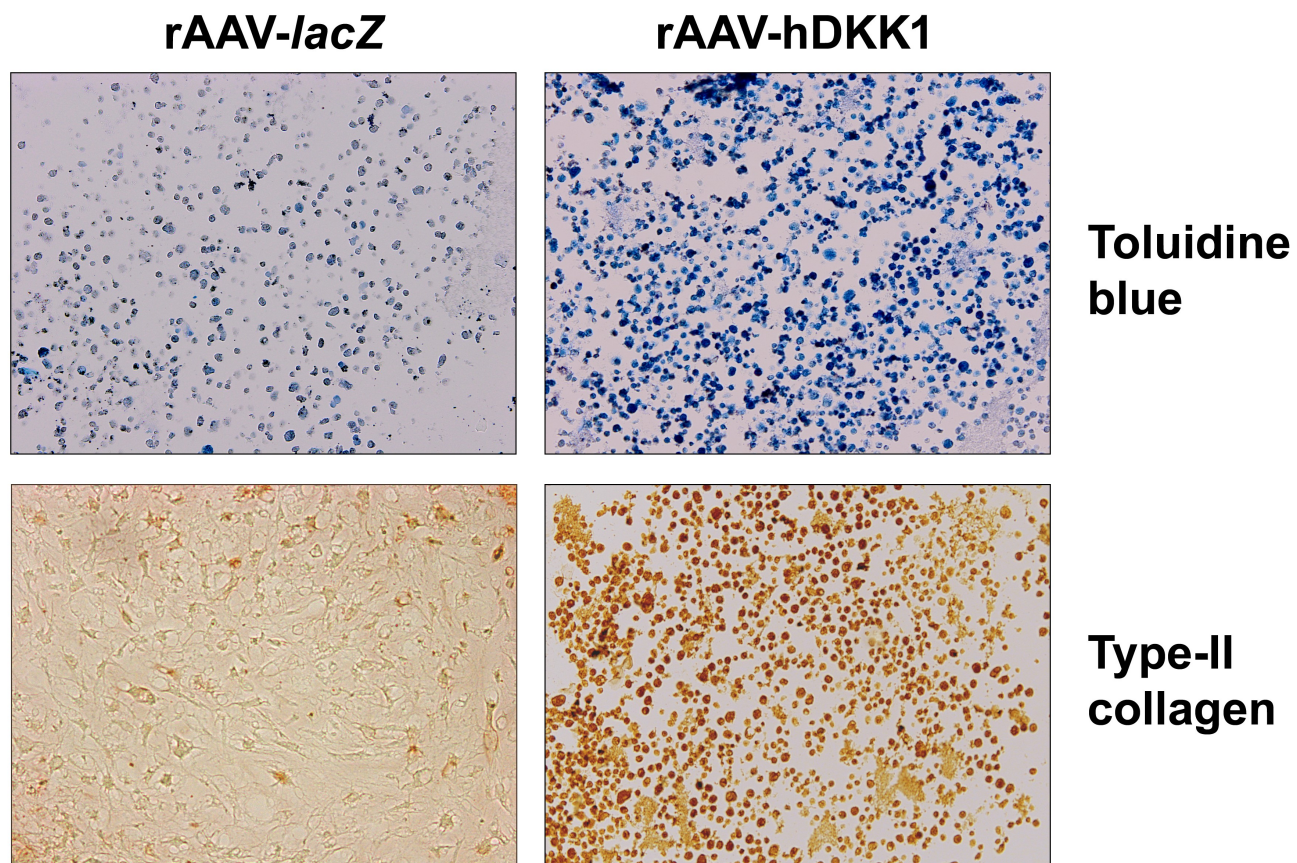


Figure 2. Metabolic and chondrogenic differentiation processes in rAAV-transduced chondrogenically-induced hMSCs. Aggregate cultures ($n = 24$ using 8 bone marrow aspirates in three independent experiments) were transduced in triplicate with rAAV-hDKK1 ($n = 72$) or rAAV-*lacZ* ($n = 72$) (40 μ l vector, MOI = 4) as described in Figure 1 and processed after 21 days for toluidine blue staining ($n = 10$ histological sections analyzed per replicate condition for each vector, i.e. $n = 30$ for rAAV-hDKK1 and $n = 30$ for rAAV-*lacZ* to represent all aggregate cultures) and immunodetection of type-II collagen ($n = 10$ immunohistochemical sections analyzed per replicate condition for each vector, i.e. $n = 30$ for rAAV-hDKK1 and $n = 30$ for rAAV-*lacZ* to represent all aggregate cultures) (magnification $\times 10$; all representative data) as described in the Materials and Methods.

($98 \pm 2\%$ transduction efficiencies) *versus lacZ* transduction ($18 \pm 2\%$, i.e. a 5.4-fold difference, $P \leq 0.001$) over a selected time (21 days) adapted to achieve hMSC chondrogenesis (Venkatesan *et al.* 2012) (Figure 1B). Also, the levels of DKK1 produced in the rAAV-hDKK1-transduced aggregates were always significantly higher than those measured in the rAAV-*lacZ*-transduced samples (up to 18-fold difference, $P \leq 0.001$) (Table 1).

Effects of DKK1 overexpression

Chondrogenically-induced aggregate cultures of hMSCs were then treated with rAAV-hDKK1 to monitor the potential effects of DKK1 overexpression on the metabolic activities and chondrogenic differentiation processes in the cells over time in this environment compared with control (rAAV-*lacZ*) treatment.

Application of the DKK1 vector significantly increased the DNA contents in the hMSC aggregate cultures after 21 days relative to *lacZ* treatment (1.2-

fold difference, $P \leq 0.001$) (Table 2). Successful chondrogenic differentiation was achieved in the aggregates after 21 days, most particularly when applying the rAAV-hDKK1 vectors *versus* control *lacZ* gene transfer as seen by the more intense toluidine blue staining and type-II collagen immunostaining (1.4- and 1.6-fold difference, respectively, $P \leq 0.001$) (Figure 2 and Table 3). Concordant with that, the proteoglycan and type-II collagen contents were significantly more elevated using the DKK1 vector compared with rAAV-*lacZ* at this prolonged time point (always 1.5-fold difference, respectively, $P \leq 0.001$) (Table 2). Furthermore, an evaluation of the proteoglycan and type-II collagen contents normalized to the proliferation indices revealed significantly higher amounts of this major matrix components with rAAV DKK1 relative to *lacZ* on day 21 (always 1.3-fold difference, respectively, $P \leq 0.001$) (Table 2). In marked contrast, the deposition of undesirable type-I and type-X collagen osteogenic and hypertrophic markers was almost completely pre-

Table 3. Histomorphometric analyses in rAAV-transduced chondrogenically-induced hMSC aggregate cultures (day 21).

Parameter	rAAV- <i>lacZ</i>	rAAV-hDKK1
Toluidine blue staining	2.7 (0.5)	3.7 (0.6)*
Type-II collagen immunostaining	2.4 (0.5)	3.9 (0.4)*
Alizarin red staining	3.4 (0.5)	2.3 (0.6)*
Type-I collagen immunostaining	3.7 (0.5)	0.4 (0.1)*
Type-X collagen immunostaining	95 (7)	2 (1)*

vented by addition of rAAV-hDKK1 over this period of evaluation compared with the elevated levels of expression of these components in the presence of rAAV-*lacZ* (9.3- and 48-fold difference, respectively, $P \leq 0.001$), together with a significant reduction of alizarin red staining (1.5-fold, $P \leq 0.001$) (Figure 3 and Table 3).

Gene expression profiles in human bone marrow-derived mesenchymal stem cells following DKK1 overexpression

hMSCs in chondrogenically-induced aggregate cultures were finally modified with rAAV-hDKK1 to examine the influence of DKK1 overexpression on the

gene expression profiles in the cells over time relative to control (rAAV-*lacZ*) treatment. As we previously reported that rAAV gene transfer does not impair hMSC chondrogenesis (Venkatesan *et al.* 2012), we did not further include here a condition without vector treatment as additional control.

Overall, the results of the real-time RT-PCR analysis corroborated the findings of the histological, immunohistochemical, and biochemical analyses. Administration of the rAAV DKK1 vector significantly increased the levels of SOX9 and COL2A1 expression over the period of evaluation (21 days) relative to the control *lacZ* condition (2.7- and 1.9-fold difference, respectively, $P \leq 0.001$) while decreasing that of

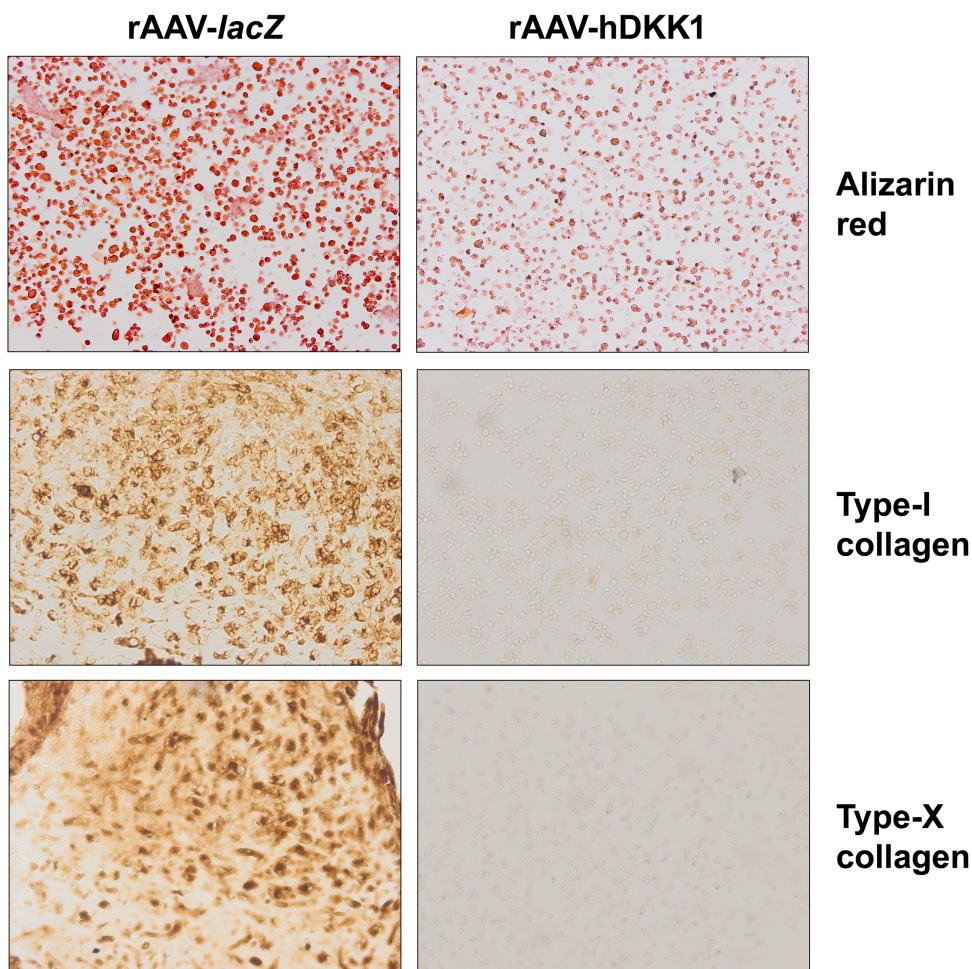


Figure 3. Hypertrophic and osteogenic differentiation processes in rAAV-transduced chondrogenically-induced hMSCs. Aggregate cultures ($n = 24$ using 8 bone marrow aspirates in three independent experiments) were transduced in triplicate with rAAV-hDKK1 ($n = 72$) or rAAV-*lacZ* ($n = 72$) (40 μ l vector, MOI = 4) as described in Figures 1 and 2 and processed after 21 days for alizarin red staining ($n = 10$ histological sections analyzed per replicate condition for each vector, i.e. $n = 30$ for rAAV-hDKK1 and $n = 30$ for rAAV-*lacZ* to represent all aggregate cultures) and immunodetection of type-I collagen ($n = 10$ immunohistochemical sections analyzed per replicate condition for each vector, i.e. $n = 30$ for rAAV-hDKK1 and $n = 30$ for rAAV-*lacZ* to represent all aggregate cultures) and of type-X collagen ($n = 10$ immunohistochemical sections analyzed per replicate condition for each vector, i.e. $n = 30$ for rAAV-hDKK1 and $n = 30$ for rAAV-*lacZ* to represent all aggregate cultures) (magnification $\times 10$; all representative data) as described in the Materials and Methods.

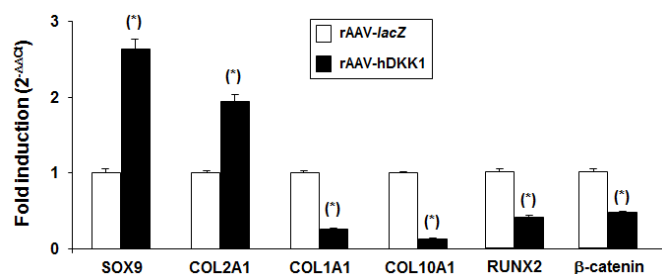


Figure 4. Gene expression analysis in rAAV-transduced chondrogenically-induced hMSCs. Aggregate cultures ($n = 24$ using 8 bone marrow aspirates in three independent experiments) were transduced in triplicate with rAAV-hDKK1 ($n = 72$) or rAAV-lacZ ($n = 72$) (40 μ l vector, MOI = 4) as described in Figures 1-3 and all the cultures and conditions were processed after 21 days to monitor the gene expression profiles of the transcription factor SOX9, type-II collagen (COL2A1), type-I collagen (COL1A1), and type-X collagen (COL10A1), the transcription factor RUNX2, and β -catenin, with GAPDH serving as a housekeeping gene and internal control. Ct values were obtained for each target gene using GAPDH as a control for normalization and fold inductions (relative to lacZ aggregates) were measured by using the $2^{-\Delta\Delta C_t}$ method as described in the Materials and Methods. *Statistically significant compared with rAAV-lacZ.

COL1A1 and COL10A1 (3.8- and 7.7-fold difference, respectively, $P \leq 0.001$), in association with reduced RUNX2 and β -catenin expression (2.4- and 2.2-fold difference, respectively, $P \leq 0.001$) (Figure 4).

Discussion

Application of genetically modified chondroregenerative MSCs may provide powerful tools to address the problem of the inappropriate formation of fibrocartilaginous tissue in sites of cartilage injury occurring during spontaneous repair and following classical or MSC-guided surgical interventions (Madry *et al.* 2010, Skowronski & Rutka 2013, Wakitani *et al.* 2004). While a number of candidate factors have been reported for their chondroregenerative potential in these cells (COMP, TGF- β , BMPs, FGF-2, IGF-I, IL-10, SOX and ZNF145 transcription factors, signaling Ihh and Wnt11 molecules, anti-Cbfa-1 siRNA) (Cucchiari *et al.* 2011, Frisch *et al.* 2014a, 2014b, Haleem-Smith *et al.* 2012, Ikeda *et al.* 2004, Jeon *et al.* 2012, Kim & Im 2011, Liu *et al.* 2011, 2014, 2015, Pagnotto *et al.* 2007, Steinert *et al.* 2009, 2012, Venkatesan *et al.* 2012), there is no evidence to date demonstrating the consistent and full regeneration of a hyaline cartilage using any of these agents *in vivo* (Cucchiari *et al.* 2011, Madry 2005). The goal of the present study therefore was to identify novel factors capable of enhancing MSC chondrogenesis *in vitro* as a first step

towards future evaluations in relevant animal models of cartilage defects *in vivo* (Cucchiari *et al.* 2011, Madry 2005, Liu *et al.* 2011, Pagnotto *et al.* 2007). We focused on delivering a genetic sequence coding for the chondroprotective DKK1 inhibitor of the Wnt signaling pathway (Funck-Brentano *et al.* 2014, Oh *et al.* 2012) via the highly potent rAAV vectors that are well adapted for clinical applications (Evans & Huard 2015, Madry & Cucchiari *et al.* 2016).

Our results first reveal that highly effective and prolonged expression of DKK1 may be obtained via rAAV gene transfer in both undifferentiated and chondrogenically-induced hMSCs *versus* control (lacZ) treatment over a period of 21 days (the longest time point examined), reaching 98% transduction efficiencies, concordant with previous findings using this class of vector (Cucchiari *et al.* 2011, Pagnotto *et al.* 2007, Venkatesan *et al.* 2012). The levels of DKK1 factor produced ranged from 0.60 ± 0.03 to 0.36 ± 0.03 pg/ml/ 10^6 cells/24 h between days 3 and 21 in undifferentiated hMSC monolayer cultures and from 0.18 ± 0.02 to 0.11 ± 0.01 pg/ml/ 10^6 cells/24 h in chondrogenically-induced hMSC aggregate cultures (i.e. an about 3.3-fold difference between systems when comparing similar time points, probably reflecting the 5-fold difference of MOIs: MOI = 20 in monolayer cultures *versus* MOI = 4 in aggregate cultures). In all cases, significant and durable levels of expression were noted relative to control treatment, most likely due to the good maintenance of the rAAV transgene in the targets (Xiao *et al.* 1996).

The data next indicate that successful DKK1 overexpression in hMSCs using rAAV led to enhanced cell proliferation levels over an extended period of time (21 days) relative to lacZ treatment, owing to the durable production of DKK1 from the construct. This is in good agreement with previous observations showing increased proliferation promoted by recombinant DKK1 (rDKK1) in such cells (Gregory *et al.* 2003) but in contrast with findings from the Im's group (Im *et al.* 2010, 2011) who reported decreased proliferation indices when applying rDKK1 to hMSCs. However, in these studies such effects were described after 6 days of treatment using high doses of factor (300 ng/ml) while Gregory *et al.* (Gregory *et al.* 2003) applied DKK1 at 100 ng/ml (i.e. 3-fold less) and we reported maximal DKK1 production levels of 0.60 pg/ml/ 10^6 cells 24 h with rAAV, overall suggesting that balancing DDK1 expression will be critical for optimal therapeutic benefits.

The current results also show that administration of rAAV-hDKK1 was capable of stimulating over time the anabolic and differentiation activities of hMSCs (proteoglycans, type-II collagen) *versus* rAAV

-lacZ, also probably resulting from the steady overexpression of DKK1 via rAAV that led to enhanced levels of chondrogenic SOX9 expression as reported by others (Im & Quan 2010). Such chondroregenerative effects of DKK1 are overall consistent with previous observations using rDKK1 to target MSCs (Im & Quan 2010; Leijten *et al.* 2012) or by indirectly blocking this factor with an anti-DKK1 antibody (Zhong *et al.* 2016).

Finally, the data demonstrate that modification of hMSCs by rAAV DKK1 gene transfer advantageously reduced unwanted hypertrophic and osteogenic events in the cells (mineralization, type-I and -X collagen), via decreased levels of RUNX2 (osteogenic transcription factor) and β -catenin (mediator of the Wnt signaling pathway for osteoblast lineage differentiation) *versus* increased expression profiles of SOX9 (a well-known factor with anti-hypertrophic activities) (Akiyama *et al.* 2004, Amano *et al.* 2009, Hattori *et al.* 2010, Leung *et al.* 2011, Venkatesan *et al.* 2012), all in good agreement with the properties of DKK1 (Fedi *et al.* 1999, Glinka *et al.* 1998, Sandell & Adler 1999) and with previous findings using rDKK1 (Gregory *et al.* 2003, Im & Quan 2010, Leijten *et al.* 2012) or by indirect anti-DKK1 neutralization (Zhong *et al.* 2016).

In conclusion, the present findings show the benefits of modifying hMSCs via rAAV to overexpress DKK1 for chondroregenerative purposes. The next step will be to translate the findings in animal cells as a preliminary approach to evaluations in clinically relevant (orthotopic) experimental models of cartilage defects *in vivo* (Cucchiariini & Madry 2005, Liu *et al.* 2011, Pagnotto *et al.* 2007) to evidence potential therapeutic and confirm the anti-hypertrophic activities of DKK1. This will be also critical to exclude any decisive impact on bone homeostasis and osteoarthritis (Krause *et al.* 2014, Mason *et al.* 2017, Weng *et al.* 2010). In this regard, a close modulation of DKK1 expression may be envisaged by tight gene regulation using tissue-specific (SOX9, type-II collagen, COMP) or regulatable promoters (tetracycline-sensitive) (Apparailly *et al.* 2002, Goldring *et al.* 1994). Overall, the current findings are valuable to further develop effective approaches for the treatment of articular cartilage lesions.

Conflicts of interest

The authors declare no conflict of interest associated with this work.

Author Contributions

MC designed the study and drafted the manuscript. JKV carried out the major experiments and contributed in manuscript writing. GS, SS-M, and HM helped in histological, immunohistochemical, and histomorphometric experiments. All authors read and approved the final version of the manuscript.

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