Effects of combined rAAV-mediated TGF-β and sox9 gene transfer and overexpression on the metabolic and chondrogenic activities in human bone marrow aspirates

Ke Tao¹,², Ana Rey-Rico², Janina Frisch², Jagadeesh Kumar Venkatesan², Gertrud Schmitt², Henning Madry²,³, Jianhao Lin¹* and Magali Cucchiarini²*

Abstract

Background: Transplantation of genetically modified bone marrow concentrates is an attractive approach to conveniently activate the chondrogenic differentiation processes as a means to improve the intrinsic repair capacities of damaged articular cartilage.

Methods: Human bone marrow aspirates were co-transduced with recombinant adeno-associated virus (rAAV) vectors to overexpress the pleiotropic transformation growth factor beta (TGF-β) and the cartilage-specific transcription factor sox9 as a means to enhance the chondroreparative processes in conditions of specific lineage differentiation.

Results: Successful TGF-β/sox9 combined gene transfer and overexpression via rAAV was achieved in chondrogenically induced human bone marrow aspirates for up to 21 days, the longest time point evaluated, leading to increased proliferation, matrix synthesis, and chondrogenic differentiation relative to control treatments (reporter lacZ treatment, absence of vector application) especially when co-applying the candidate vectors at the highest vector doses tested. Optimal co-administration of TGF-β with sox9 also advantageously reduced hypertrophic differentiation in the aspirates.

Conclusions: These findings report the possibility of directly modifying bone marrow aspirates by combined therapeutic gene transfer as a potent and convenient future approach to improve the repair of articular cartilage lesions.

Keywords: Human bone marrow aspirates, Recombinant adeno-associated virus, Combined gene transfer, Transforming growth factor beta, SOX9, Chondrogenesis

Background

Articular cartilage over the surface of epiphysis of bone is a highly specialized connective tissue formed by chondrocytes, enabling almost smooth, frictionless movement between the articulating surfaces of diarthrodial joints. Due to its intrinsic limited potential for self-healing, damaged articular cartilage is not capable of restoring its original structure and functions and often degenerates towards osteoarthritis. Currently available therapeutic options in the clinics have increasingly focused on cell-based approaches including bone marrow stimulation techniques (microfracture, pridie drilling, abrasion) that promote the penetration of chondroregenerative cells from the subchondral bone marrow in sites of cartilage lesions (Madry et al. 2010). Yet, such procedures do not allow to reproduce the original cartilage, allowing only for the production of a fibrocartilaginous repair tissue made of type-I collagen and of poor mechanical quality, but not of the native, highly organized hyaline cartilage (proteoglycans, type-II collagen) capable of supporting joint loading and motion (Breinan et al. 2000; Frisbie et al. 2003; Madry et al. 2010; Orth et al. 2014).

While administration of human bone marrow-derived mesenchymal stem cells (hMSCs) has been already attempted in patients to activate the regenerative
processes in focal cartilaginous lesions (Haleem et al. 2010; Kuroda et al. 2007; Nejadnik et al. 2010; Orth et al. 2014; Skowronska and Rutka 2013; Wakitani et al. 2004; Wakisaka et al. 2007), the necessity of applying multiple steps to harvest the cells and propagate them over time in vitro has weakened its widespread clinical application. Application of bone marrow concentrates containing chondrogenically competent MSCs among other cell populations (hematopoietic cells, fibroblast-like cells) in a natural biochemical (growth factors) microenvironment instead may provide less demanding approaches for the goal of therapeutic transplantation in cartilage defects (Gigante et al. 2012; Kim et al. 2014; Slynarski et al. 2006) as also performed to treat femoral head necrosis and nonunion bone fractures (Hauzeur and Gangji 2010; Kon et al. 2012). Still, even though encouraging results have been obtained when implanting such samples in preclinical models and in patients, the current outcomes have not been consistently associated with the formation of a functional, hyaline-like repair tissue that fully and stably integrates with the surrounding, intact cartilage (Gigante et al. 2012; Ivkovic et al. 2010; Kim et al. 2014; Pascher et al. 2004; Slynarski et al. 2006).

In this regard, genetic modification of bone marrow concentrates by transfer of sequences coding for chondrogenic and/or chondroreparative factors prior to implantation in sites of cartilage injury might be a powerful tool to overcome such critical issues (Frisch et al. 2015; Johnstone et al. 2013). Various therapeutic sequences have been tested to achieve this goal, including the cartilage oligomeric matrix protein (COMP), bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β), insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (FGF-2), the sex-determining region Y-type high-mobility group box (SOX) family of transcription factors, zinc finger protein 145 (ZNF145), Indian hedgehog (Ihh), Wnt11, and small interfering RNA (siRNA) against p53 and runt-related transcription factor 2 (Runx2) (Babister et al. 2008; Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Haleem-Smith et al. 2012; Huang et al. 2010; Ikeda et al. 2004; Jeon et al. 2012; Kim and Im 2011; Lee et al. 2011; Liu et al. 2011; Liu et al. 2013; Liu et al. 2014; Liu et al. 2015; Neumann et al. 2013; Pagnotto et al. 2007; Steinert et al. 2009; Steinert et al. 2012; Tao et al. 2016; Venkatesan et al. 2012). Interestingly, previous reports from diverse groups, among which ours, demonstrated that multiple therapeutic gene transfer might be necessary to promote optimal reparative activities in various cells of the musculoskeletal system (articular chondrocytes, MSCs) (Cucchiarini et al. 2009; Ikeda et al. 2004; Kim and Im 2011; Liu et al. 2013; Liu et al. 2015; Steinert et al. 2009; Tao et al. 2016).

In light of our work showing the therapeutic benefits of overexpressing TGF-β simultaneously with sox9 in isolated human MSCs (Tao et al. 2016) and independently in human bone marrow aspirates (Frisch et al. 2016; Rey-Rico et al. 2015) without any interference of independent vectors in dual versus single gene transfer, we tested here the possibility of co-delivering these two highly chondrogenic factors to further enhance the repair processes in primary human bone marrow aspirates. We specifically focused on gene transfer using the clinically adapted recombinant adeno-associated virus (rAAV) vectors that can transduce MSCs at very high efficiencies (up to 100%) and over extended periods of time (at least 3 weeks) without altering their differentiation potential (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Lee et al. 2011; Pagnotto et al. 2007; Tao et al. 2016; Venkatesan et al. 2012). Of further note, transduction via rAAV does not raise viral interference, allowing for concomitant administration of independent vectors in their targets (Cucchiarini et al. 2009).

For the first time to our best knowledge, we provide evidence that successful, prolonged co-overexpression of TGF-β and sox9 using this vector class synergically enhances the levels of proliferation, biosynthesis, and chondrogenesis in human bone marrow concentrates relative to control conditions (reporter lacZ treatment, absence of vector application) while delaying undesirable hypertrophic and osteogenic differentiation. These observations support the concept of modifying bone marrow aspirates by multiple rAAV vectors as a promising approach for future implantation procedures in articular cartilage defects in vivo.

Methods

Chemicals and reagents

All reagents were purchased at Sigma (Munich, Germany) unless otherwise indicated. The dimethylmethylene blue dye was from Serva (Heidelberg, Germany). Recombinant TGF-β3 was from Peprotech (Hamburg, Germany). The antibodies used for immunohistochemical analyses were as follows: the anti-β-galactosidase (β-gal) (GAL-13) and anti-type-X collagen (COL-10) antibodies from Sigma, the anti-TGF-β (V), anti-SOX9 (C-20), and anti-FLAG tag (BioM2) antibodies from Santa Cruz Biotechnology (Heidelberg, Germany), the anti-type-I collagen (COL-1) antibody from Abcam (Cambridge, UK), and the anti-type-II collagen (II-IIB3, NIH Hybridoma Bank, University of Iowa, Ames, USA) antibody from Acris (Hiddenhausen, Germany). Biotinylated secondary antibodies and the ABC reagent were purchased at Vector Laboratories (Alexis Deutschland GmbH, Grünberg, Germany). The TGF-β enzyme-linked immunosorbent assay (active hTGF-β1 Quantikine ELISA) was from R&D Systems (Wiesbaden, Germany).
Human bone marrow aspirates

Human bone marrow aspirates (~15 ml; 1.4 ± 0.4 × 10^9 cells/ml) were obtained from the distal femur of osteoarthritic patients undergoing total knee arthroplasty (n = 8, age 69 ± 11 years) (Frisch et al. 2016; Rey-Rico et al. 2015). All patients provided informed consent before inclusion in the study and all procedures were in accordance with the Helsinki Declaration. The study was approved by the Ethics Committee of the Saarland Physicians Council (Application 06/08).

Plasmids and rAAV vectors

The parental AAV-2 genomic clone pSSV9 was used to create all constructs applied in this study (Samulski et al. 1987; Samulski et al. 1989). rAAV-lacZ carries the lacZ gene for E. coli β-galactosidase under the control of the cytomegalovirus immediate-early (CMV-IE) promoter. rAAV-hTGF-β carries a 1.2-kb human transforming growth factor beta 1 (hTGF-β1, active form) cDNA fragment and rAAV-FLAG-hsox9 a 1.7-kb FLAG-tagged human sox9 (hsox9) cDNA, both cloned in rAAV-lacZ in place of lacZ (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Frisch et al. 2016; Rey-Rico et al. 2015; Tao et al. 2016; Venkatesan et al. 2012). rAAV were packaged as conventional (not self-complementary) vectors using the 293 adenovirus-transformed embryonic kidney cell line. Adenovirus 5 was used to provide helper functions in combination with the pAd8 helper plasmid as previously described (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Frisch et al. 2016; Rey-Rico et al. 2015; Tao et al. 2016; Venkatesan et al. 2012). The vectors were purified, dialyzed, and titrated by real-time PCR (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Frisch et al. 2016; Rey-Rico et al. 2015; Tao et al. 2016; Venkatesan et al. 2012), vascularizing 10^10 transgene copies/ml (ratio virus particles to functional vectors = 500/1).

rAAV-mediated gene transfer

Aspirates were aliquoted in standard tissue culture plastic 96-well plates (100 μl of aspirate/well) and immediately transduced with the rAAV vectors (rAAV-lacZ; 20 or 40 μl) or co-transduced (rAAV-hTGF-β/rAAV-FLAG-hsox9; 10 or 20 μl each vector) with each aliquot (8 × 10^5 functional recombinant viral particles, MOI = 10 ± 3) (Frisch et al. 2016; Rey-Rico et al. 2015), while aspirates were added only 40 μl DMEM medium as the negative control group. A volume of 60 μl of chondrogenic medium (4.5 g/l DMEM high glucose, 100 U/ml penicillin and 100 μl/ml streptomycin, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenious acid, 5.35 μg/ml linoleic acid, 1.25 μg/ml BSA, 1 mM sodium pyruvate, 37.5 μg/ml ascorbate 2-phosphate, 10^{-7} M dexamethasone, 10 ng/ml TGF-β3) was then added per aspirate with 250 μl of the abovementioned medium change performed once per week for MSC chondrogenesis for up to 21 days (Barry et al. 2001; Frisch et al. 2016; Johnstone et al. 1998; Rey-Rico et al. 2015; Yoo et al. 1998). To avoid attachment on the bottom of the plates, the aspirates were carefully mixed after each medium change. For osteogenic and adipogenic differentiation, the aspirates were transduced using similar rAAV-mediated gene transfer conditions as those described above and then induced either toward osteogenic differentiation using the StemPro Osteogenesis Differentiation kit or adipogenic differentiation using the StemPro Adipogenesis Differentiation kit (Life Technologies GmbH, Darmstadt, Germany) (Frisch et al. 2016).

Detection of transgene expression

To assess TGF-β secretion, 30 μl of culture supernatant were collected at the denoted time points 24 h after medium change and centrifuged to remove debris and TGF-β production was measured by ELISA as previously described (Frisch et al. 2016). Quantitative measurements were performed on a GENios spectrophotometer/fluorometer (Tecan, Crailsheim, Germany). Moreover, after 21 days, the aspirates were collected by centrifugation at 1,500 rpm/min for 5 min to form pellet and subsequently fixed in 4% formalin, dehydrated in graded alcohols, embedded in paraffin, and sectioned (3 μm). Transgene expression (lacZ, TGF-β, sox9, FLAG) was also assessed by immunohistochemical analyses using specific primary antibodies, biotinylated secondary antibodies, and the ABC method with diaminobenzidine as the chromogen (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Frisch et al. 2016; Rey-Rico et al. 2015; Tao et al. 2016; Venkatesan et al. 2012). To control for secondary immunoglobulins, the samples were processed with omission of the primary antibody. Samples were examined under light microscopy (Olympus BX 45) (Olympus, Hamburg, Germany).

Histological, immunocytochemical, and immunohistochemical analyses

Aspirates were collected and centrifuged to form pellet, fixed in 4% formalin, dehydrated in graded alcohols, embedded in paraffin, and sectioned (3 μm). Sections were stained with hematoxylin eosin (H&E) for cellularity, toluidine blue for matrix proteoglycans, and alizarin red for matrix mineralization (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Frisch et al. 2016; Rey-Rico et al. 2015; Tao et al. 2016; Venkatesan et al. 2012). Immunohistochemical analyses were performed to monitor the expression of type-II, -I, and -X collagen using specific primary antibodies, biotinylated secondary antibodies, and the ABC method with
diaminobenzidine as the chromogen (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Frisch et al. 2016; Rey-Rico et al. 2015; Tao et al. 2016; Venkatesan et al. 2012). To control for secondary immunoglobulins, sections were processed with omission of the primary antibody. Samples were examined under light microscopy (Olympus BX 45).

**Morphometric analyses**

The levels of cells expressing the transgene (% of β-gal+, cells), the cell densities on H&E-stained sections, the densities of toluidine blue and alizarin red staining and those of type-II, -I, and -X collagen, TGF-β, and SOX9 immunostaining were monitored at three random standardized sites or with 10 serial histological and immunohistochemical sections for each parameter, test and replicate condition using the SIS analySIS program (Olympus), Adobe Photoshop (Adobe Systems, Unterschleissheim, Germany), and Scion Image (Scion Corporation, Frederick, MD, USA) (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Frisch et al. 2016; Rey-Rico et al. 2015; Tao et al. 2016; Venkatesan et al. 2012). To evaluate β-gal expression, only cells strongly stained versus (faint) background DAB signal, i.e. in the absence of primary antibody, were considered as β-gal+. Regarding the measurements of the cell densities, H&E-stained sections from either lacZ, TGF-β-, and sox9-treated aspirates were analyzed by counting cells per standardized area using a similar magnification (×40) for all types of samples in order to allow for strict comparison. To monitor the staining intensities, x20 images were first converted to inverted grayscale mode. Background DAB signal was adapted for comparable range of each type of immunostaining prior to evaluations. The total areas (mm²) covered with cells were next measured to identify the average gray value of the defined area. Data are given as mean intensity of staining or immunostaining per mm² of total cell area.

**Biochemical assays**

The aspirates were collected and digested with papain as previously described (Frisch et al. 2016). A fluorimetric assay using Hoechst 22358 was applied to determine the DNA contents and the proteoglycans were measured by binding to dimethylmethylene blue dye (Cucchiarini et al. 2009; Cucchiarini et al. 2011; Frisch et al. 2014; Frisch et al. 2016; Rey-Rico et al. 2015; Tao et al. 2016; Venkatesan et al. 2012). Total cellular proteins were monitored via protein assay (Pierce Thermo Scientific, Fisher Scientific, Schwerte, Germany) and subsequently used to normalize the biochemical data. All measurements were performed on a GENios spectrophotometer/fluorometer (Tecan).

**Real-time RT-PCR analyses**

Total cellular RNA was extracted from all the aspirates at the denoted time points using TRIzol reagent (Ambion® Life Technologies) and the RNeasy Protect Mini Kit with an on-column RNase-free DNase treatment (Qiagen, Hilden, Germany) and RNA elution in 30 μl RNase-free water. 8 μl of eluate were applied to perform reverse transcription by using the 1st Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Applied Science). Real-time PCR was carried out to amplify 2 μl of the cDNA product by using the Brilliant SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Waldbronn, Germany) on an Mx3000P QPCR operator system (Stratagene) as follows: (95 °C, 10 min), amplification by 55 cycles (denaturation at 95 °C, 30 s; annealing at 55 °C, 1 min; extension at 72 °C, 30 s), denaturation (95 °C, 1 min), and final incubation (55 °C, 30 s) (Frisch et al. 2016). All primers were purchased at Invitrogen (Darmstadt, Germany): SOX9 (chondrogenic marker) (forward 5′-ACACACAGCTCACCTGACCTTG-3’; reverse 5′-GGGAATTCTGGTGTTGCTCTT-3’), aggrecan (ACAN) (chondrogenic marker) (forward 5′-GAGATGGAGGTTGAGGTC-3′; reverse 5′-AGCTGCTCTGGGCCTTC-3′), type-II collagen (COL2A1) (chondrogenic marker) (forward 5′-GGACTTTTCTCCTCCTTCT-3′; reverse 5′-GACCCTAGGGTGTTACAGGA-3′), type-I collagen (COL1A1) (osteogenic marker) (forward 5′-AGCTTCTGGAAGTGGTGC-3′; reverse 5′-GACCCAGAGTTCTTACAGGA-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene and internal control) (forward 5′-CCTCTTTGTATGTGCCAACC-3′; reverse 5′-AGATTCAGTCCCTGGTGTA-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene and internal control) (forward 5′-GAAGTTGGTC-3′; reverse 5′-GAAGATGTTGATGGATTTC-3′) (all 150 nM final concentration) (Frisch et al. 2016). Water and non-reverse-transcribed mRNA were used as control conditions. To confirm the specificity of the products, melting curve analysis and agarose gel electrophoresis were performed. The threshold cycle (Ct) value for each amplified sample and each gene of interest was measured by using the MxPro QPCR software (Strata- gene), and values were normalized to GAPDH expression by using the 2-ΔΔCt method (Frisch et al. 2016).

**Statistical analyses**

All conditions were performed in triplicates in three independent experiments for each patient and all patients were tested. Data are given as mean ± standard deviation (SD) of separate experiments. Statistical significance was assessed for any p value of less than 0.05 using the t-test and Mann–Whitney Rank Sum Test where appropriate.
Results
Effective and sustained TGF-β and sox9 combined gene transfer and overexpression via rAAV in human bone marrow aspirates

Bone marrow aspirates were first transduced with the combination rAAV-hTGF-β/rAAV-FLAG-hsox9 to evaluate the ability of rAAV to mediate direct co-overexpression of TGF-β and sox9 over time in aspirates committed toward the chondrogenic differentiation compared with control conditions (rAAV-lacZ transduction, absence of vector treatment). As we previously reported that rAAV is capable of successfully expressing functional TGF-β and SOX9 factors separately in human bone marrow aspirates (Frisch et al. 2016; Rey-Rico et al. 2015), we did not include single gene treatments here as additional controls.

An evaluation of the % of the β-gal⁺ cells revealed higher transduction efficiencies in aspirates transduced with rAAV-lacZ after 21 days compared with the other conditions (2.7- to 3.7-fold difference at 20 or 40 μl vector, respectively, p ≤ 0.010) (Table 1) as estimated on immunohistochemical sections from aspirates (Fig. 1a). Strong, significant TGF-β expression was also observed in the aspirates after 21 days especially when rAAV-hTGF-β was provided to the aspirates as noted by immunohistochemical analysis that revealed the strongest signal upon concomitant TGF-β and sox9 gene transfer at the highest vector doses applied (20 μl each vector) relative to rAAV-lacZ and to untreated aspirates (Fig. 1a). These results were corroborated by the results of an TGF-β ELISA (up to 1.9-fold higher amounts of TGF-β produced when co-applying the TGF-β and sox9 vectors at the highest vector doses versus control conditions, p ≤ 0.050) (Fig. 1b). Strongest sox9 expression was also observed in aspirates modified by rAAV-FLAG-hsox9/rAAV-hTGF-β at the highest vector doses while expression of the FLAG tag was only detected when applying the sox9 vector (Fig. 1a).

Table 1 Evaluation of the % of β-gal⁺ cells in rAAV-transduced human bone marrow aspirates (day 21)

| Condition                  | % of β-gal⁺ cells |
|----------------------------|-------------------|
| no vector                  | 21.6 (0.8)        |
| lacZ (20 μl)               | 54.5 (2.8) ab     |
| lacZ (40 μl)               | 75.1 (1.9) ab     |
| TGF-β + sox9 (10 μl + 10 μl)| 20.9 (1.8)        |
| TGF-β + sox9 (20 μl + 20 μl)| 20.3 (2.1)        |

Data are given as mean (SD). Statistically significant compared with *TGF-β + sox9* and *(no vector)* conditions (p ≤ 0.010)

Activation of the proliferative, biosynthetic, and chondrogenic activities in human bone marrow aspirates upon co-overexpression of TGF-β and sox9 via rAAV vectors

We next examined whether rAAV-mediated TGF-β and sox9 co-overexpression was capable of modulating the proliferative, metabolic, and differentiation activities in human bone marrow aspirates maintained over time under chondrogenic stimulation relative to control conditions (rAAV-lacZ, no vector treatment).

High, significant levels of proliferation were noted when co-delivering the TGF-β and sox9 vectors to the aspirates for 21 days especially at the highest vector doses compared with the other conditions as noted by histomorphometric evaluations performed on H&E-stained sections from aspirates (up to 3.2-fold difference for both the H&E staining intensities and cell densities, p ≤ 0.010) (Fig. 2a-c), a finding corroborated when estimating the DNA contents in the samples (up to 2.7-fold difference, p ≤ 0.010) (Fig. 2d). Elevated, significant levels of matrix synthesis and chondrogenic differentiation were also achieved upon TGF-β and sox9 co-gene transfer in the aspirates after 21 days particularly at the highest vector doses relative to the other conditions as observed by histomorphometric evaluations performed on histological sections from aspirates stained for toluidine blue and type-II collagen (up to 7.5- and 3.8-fold difference for the toluidine blue staining intensities and for those of type-II collagen immunostaining, respectively, p ≤ 0.010) (Fig. 3a-c), a finding supported by the results of an estimation of the proteoglycan contents in the samples (up to 2.5-fold difference, p ≤ 0.010) (Fig. 3d). Overall, these findings were corroborated by the results of a real-time RT-PCR analysis revealing enhanced levels of chondrogenic SOX9, ACAN, and COL2A1 expression upon concomitant TGF-β and sox9 gene delivery in the aspirates after 21 days particularly when providing vectors at the highest vector doses compared with the other conditions (up to 8.9-, 30-, and 2.6-fold difference for SOX9, ACAN, and COL2A1, respectively, p ≤ 0.010) (see Fig. 5).

Effects of rAAV-mediated TGF-β and sox9 co-overexpression on the hypertrophic and terminal differentiation processes in human bone marrow aspirates

We finally evaluated the potential effects of TGF-β and sox9 co-overexpression via rAAV on the occurrence of hypertrophic events in chondrogenically induced human bone marrow aspirates over time compared with control conditions (rAAV-lacZ, no vector treatment).
Remarkably, co-application of rAAV-hTGF-β/rAAV-FLAG-hsox9 at the highest vector doses reduced the levels of hypertrophy and terminal differentiation in the aspirates after 21 days compared with the other conditions as noted by histomorphometric evaluations performed on histological sections from aspirates stained with alizarin red, type-I, and -X collagen (up to 1.5-, 1.2-, and 1.3-fold difference for the alizarin red staining intensities and for those of type-I and -X collagen immunostaining, respectively, \( p \leq 0.010 \)) (Fig. 4a-d). These results were again supported by findings of a real-time RT-PCR analysis, showing lower levels of COL1A1 and COL10A1 expression using TGF-β and sox9 at the highest vector doses after 21 days relative to the other conditions (up to 15- and 11.2-fold difference for COL1A1 and COL10A1, respectively, \( p \leq 0.050 \)) (Fig. 5).

**Discussion**

As a novel, single-step approach to treat articular cartilage lesions, direct transplantation of bone marrow concentrates has the advantage of eliminating the complex and invasive preparation and expansion of progenitor cells to treat articular cartilage lesions (Johnstone et al. 2013; Orth et al. 2014). Yet, while already used in the clinics, such a procedure has not allowed to reproduce a functional hyaline cartilage in patients and instead the repair tissue formed in treated lesions remains of low quality (Gigante et al. 2012; Kim et al. 2014; Slynarski et al. 2006). Chondrogenic modification of such aspirates especially using the clinically adapted rAAV gene transfer vectors prior to re-implantation in the site of injury might be a potent tool to improve the processes of cartilage repair (Frisch et al. 2015). Here, we evaluated the potential benefits of concomitantly overexpressing the chondrogenic TGF-β and sox9 factors using multiple rAAV vectors in human bone marrow aspirates with respect to their stimulatory effects as independent treatments.
in similar samples (Frisch et al. 2016; Rey-Rico et al. 2015) or as a combination in isolated human MSCs (Tao et al. 2016).

Our results first indicate that co-overexpression of TGF-β and sox9 via rAAV can be successfully achieved at very high levels (~75% transduction efficiencies) in the aspirates over prolonged periods of time (at least 21 days), in good agreement with our previous observations in such samples (Rey-Rico et al. 2015). For comparison, other groups demonstrated the possibility of transducing rabbit and sheep aspirates with more immunogenic adenoviral vectors but with much higher vector doses (10^{10} adenoviral particles per 250 μl of aspirate versus 8 × 10^{7} rAAV particles per 100 μl here, i.e. a 5 × 10^{3}-fold difference) (Ivkovic et al. 2010; Pascher et al. 2004). Of further note, combined TGF-β/sox9 gene transfer (especially at the highest vector doses employed) allowed for the sustained expression of SOX9 as previously noted with single rAAV-FLAG-hsox9 transduction (Rey-Rico et al. 2015) and to a durable production of TGF-β relative to the control conditions, in the range of those achieved when providing rAAV-hTGF-β alone (Frisch et al. 2016). While early on, all types of cells forming the aspirates (MSCs, hematopoietic cells, fibroblast-like cells) might be permissive to genetic modification, over time mostly MSCs may promote rAAV-mediated overexpression of the transgenes when continuously maintained under MSC-specific chondrogenic culture conditions (Frisch et al. 2016).

The present data further show that prolonged, effective co-overexpression of TGF-β and sox9 led to increased levels of cell proliferation, matrix biosynthesis, and chondrogenic differentiation in the
Aspirates over time (at least 21 days), concordant with the properties of these factors (Babister et al. 2008; Bi et al. 1999; Cucchiarini et al. 2009; Frisch et al. 2014; Ikeda et al. 2004; Johnstone et al. 1998; Kim and Im 2011; Lee et al. 2011; Pagnotto et al. 2007; Steinert et al. 2009; Tao et al. 2016; Venkatesan et al. 2012) and with our previous work using independent gene transfer in similar samples (Frisch et al. 2016; Rey-Rico et al. 2015), and with effects in the range of those achieved when co-delivering these factors to isolated human MSCs (Tao et al. 2016). Again, such effects over time might be attributed to modified MSCs under continuous chondrogenic stimulation, while early on, other potentially transduced cell types may be active via paracrine effects (Frisch et al. 2016).

Equally important, combined TGF-β/sox9 transduction advantageously delayed premature hypertrophic differentiation in the aspirates versus control treatments, probably due to the effective, sustained overexpression of SOX9, a well-known anti-hypertrophic factor (Rey-Rico et al. 2015; Venkatesan et al. 2012) that may counterbalance possible hypertrophic effects of TGF-β (Barry et al. 2001; Frisch et al. 2014; Yoo et al. 1998).

Overall, we provide notable evidence of the potential of multiple gene-based approaches as a means to stimulate the chondroreparative activities in human bone marrow aspirates in order to generate practical, direct systems for implantation in sites of articular cartilage injury. Work is ongoing using

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**Fig. 3** Biosynthetic activities and chondrogenic differentiation processes in human bone marrow aspirates co-transduced with rAAV-hTGF-β/rAAV-FLAG-hsox9. Aspirates were treated and maintained in culture as described in Figs. 1 and 2. The samples were processed after 21 days to evaluate the production of (a) matrix proteoglycans (toluidine blue staining) and type-II collagen (immunostaining) (original magnification x20, bar = 100 μm, insets at x40, all representative data) with histomorphometric analyses of the intensities of toluidine blue staining (b) and of type-II collagen immunostaining (c), and (d) by estimating the proteoglycan contents in the aspirates as described in the Materials and Methods. *, ** Statistically significant compared with control groups (rAAV-lacZ, no vector condition) (*p ≤ 0.050, **p ≤ 0.010)
samples from animal species (minipigs, sheep) in order to adapt the current strategy for administration in clinically relevant, orthotopic models of focal cartilage defects that provide the natural biochemical and biomechanical microenvironment for chondrogenesis (Cucchiarini et al. 2005; Ivkovic et al. 2010; Pascher et al. 2004). Such work may confirm the therapeutic activities of dual TGF-β/sox9 gene transfer via rAAV and the advantageous delay in hypertrophic differentiation in vivo as mediated here by SOX9 in vitro (as discrepancies may occur in vivo) to counteract possible deleterious effects of TGF-β occurring upon direct gene transfer (osteophyte formation, fibrogenesis) (Bakker et al. 2001; Mi et al. 2003).

**Conclusion**

The present study describes novel, convenient strategies via rAAV-mediated multiple gene transfer to modulate the chondrogenic differentiation processes in human bone marrow concentrates for the treatment of damaged articular cartilage. Gene transcription efficiencies, cell proliferation, matrix biosynthesis, and chondrogenic differentiation activities were systematically analyzed and compared over time in aspirates among all distinct subgroups, revealing that co-overexpression of TGF-β with SOX9 via rAAV effectively and durably enhanced such key chondroregenerative activities. Evaluation in relevant, preclinical models is ongoing, requesting to extensively translate the current procedure in animal
samples, in order to determine the benefits (and monitor potential hurdles) of the approach in vivo prior to safe translation in the clinics. Additional work in human samples will be also further needed taking into account the impact of age, gender, and patient pathology on the bone marrow aspirate responses to the current treatment.

Abbreviations
ACAN: Aggrecan; BMP: Bone morphogenetic protein; cdNA: Complementary deoxyribonucleic acid; CMV-E: Cytomegalovirus immediate-early; COL10A1: Type-X collagen; COL1A1: Type-I collagen; COL2A1: Type-II collagen; COMP: Cartilage oligomeric matrix protein; Ct: Threshold cycle; DMEM: Dulbecco’s modified Eagle’s medium; ELISA: Enzyme-linked immunosorbent assay; FGF-2: Basic fibroblast growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; H&E: Hematoxylin and eosin; IGF-I: Insulin-like growth factor I; Ihh: Indian hedgehog; MSCs: Mesenchymal stem cells; PCR: Polymerase chain reaction; rAAV: Recombinant adeno-associated virus; RNA: Ribonucleic acid; TGF-β: Transforming growth factor beta; Wnt11: Wingless/Int 11; ZNF145: Zinc finger protein 145

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Authors’ contributions
KT designed the experiments, carried out the analysis, interpreted the results, and wrote the manuscript. ARR, JF, JKV, GS, and HM carried out and assisted with the analysis of the results. JL co-supervised the analysis of the results and coordinated the manuscript preparation. MC coordinated the research, co-supervised the analysis of the results, and coordinated the manuscript preparation. All authors were responsible for final approval of the article.

Competing interests
The authors declare that they have no competing interests.

Author details
1Institute of Arthritis, Peking University People’s Hospital, No. 11 Xizhimen Nan Road, Xicheng District, Beijing 100044, People’s Republic of China. 2Center of Experimental Orthopaedics, Saarland University Medical Center, Kirbingerstr. Bldg 37, D-66421 Homburg/Saar, Germany. 3Department of Orthopaedic Surgery, Saarland University Medical Center, Homburg/Saar, Germany.

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