TGF-β gene transfer and overexpression via rAAV vectors stimulates chondrogenic events in human bone marrow aspirates

Janina Frisch a, Ana Rey-Rico a, Jagadeesh Kumar Venkatesan a, Gertrud Schmitt a, Henning Madry a, b, Magali Cucchiarini a, *

a Center of Experimental Orthopaedics, Saarland University Medical Center, Homburg/Saar, Germany
b Department of Orthopaedic Surgery, Saarland University Medical Center, Homburg/Saar, Germany

Received: September 21, 2015; Accepted: December 1, 2015

Abstract

Genetic modification of marrow concentrates may provide convenient approaches to enhance the chondrogenic differentiation processes and improve the repair capacities in sites of cartilage defects following administration in the lesions. Here, we provided clinically adapted recombinant adeno-associated virus (rAAV) vectors to human bone marrow aspirates to promote the expression of the potent transforming growth factor beta (TGF-β) as a means to regulate the biological and chondrogenic activities in the samples in vitro. Successful TGF-β gene transfer and expression via AAV was reached relative to control (lacZ) treatment (from 511.1 to 16.1 pg rhTGF-β/mg total proteins after 21 days), allowing to durably enhance the levels of cell proliferation, matrix synthesis, and chondrogenic differentiation. Strikingly, in the conditions applied here, application of the candidate TGF-β vector was also capable of reducing the hypertrophic and osteogenic differentiation processes in the aspirates, showing the potential benefits of using this particular vector to directly modify marrow concentrates to generate single-step, effective approaches that aim at improving articular cartilage repair in vivo.

Keywords: cartilage repair • gene therapy • bone marrow aspirates • rAAV • TGF-β

Introduction

The adult articular cartilage has a limited ability for self-repair [1] as a result of the absence of vascularization in this highly specialized tissue. Options to provide reparative cells in sites of cartilage lesions include autologous chondrocyte implantation and marrow-stimulating techniques to recruit chondrogenic bone marrow-derived mesenchymal stem cells (MSCs) [2–4] but the outcome of such procedures do not meet the original hyaline cartilage as the repair tissue is commonly made of a fibrocartilaginous structure (type-I collagen instead of type-II collagen proteoglycans) of lesser mechanical quality and that does not integrate with the adjacent, original cartilage [5]. A promising approach might be to conveniently supply the lesions with bone marrow concentrates [6] composed of chondrogenically competent MSCs (~1%) that specifically commit towards the chondrocyte phenotype under adapted treatment conditions among other cell populations (haematopoietic progenitor cells, haematopoietic cells, fibroblasts) [7, 8] rather than using isolated progenitors that necessitate more complex steps of preparation and expansion [6]. Yet, despite encouraging attempts to treat patients with bone marrow concentrates [9–15], restoration of the articular cartilage tissue in its full integrity has not been reported to date, showing the urgent need for novel, effective regimens.

Direct genetic modification of bone marrow aspirates to overexpress chondrogenic and/or chondroreparative factors may offer improved tools to enhance the healing response in sites of cartilage injury upon local transplantation [16–18]. In marked contrast with the substantial information available on gene transfer to target isolated MSCs, little is known on the possibility to transduce marrow concentrates as a less invasive approach for improved cartilage repair. While various groups reported the ability of adenoviral vectors to mediate gene transfer in rabbit and sheep models [19–21], showing effective but relatively short-term transgene (transforming growth factor beta – TGF-β, bone morphogenetic protein 2 – BMP-2, Indian hedgehog – IHH) expression levels (some days) at high (10¹⁰–10¹¹) vector doses, we recently tested the potential of applying recombinant vectors derived from the human non-pathogenic adeno-associated virus (AAV) to human marrow aspirates in the light of the reduced toxicity and immunogenicity of this vector class that can be maintained over

*Correspondence to: Magali CUCCHIARINI
E-mail: mmcucchiarini@hotmail.com

doi: 10.1111/jcmm.12774

© 2016 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
Materials and methods

Reagents

All reagents were from Sigma-Aldrich (Munich, Germany), unless otherwise identified. Recombinant TGF-β (rTGF-β) was purchased at Peprotech (Hamburg, Germany) and the dimethylmethylene blue dye at Serva (Heidelberg, Germany). The anti-TGF-β (V) and anti-SOX9 (C-20) antibodies were from Santa Cruz Biotechnology (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 (AF-5610) antibody from the NIH Hybridoma Bank (University of Iowa, Ames, USA), and the anti-TGF-β antibody, a biotinylated secondary antibody, and diaminobenzidine as a chromogen (ABC method) [28, 31]. Our results show that administration of rAAV promotes an effective, significant production of TGF-βb, leading to enhanced levels of proliferation, biosynthesis and chondrogenic differentiation in the aspirates while reducing hypertrophy/terminal differentiation and osteo-/adipogenic events, showing the value of applying such samples in site of cartilage damage during transplantation protocols.

Plasmids and rAAV vectors

All plasmids are based on the same parental AAV-2 genomic clone, pSSV9 [29, 30]. rAAV-lacZ carries the lacZ gene encoding the Escherichia coli β-galactosidase (β-gal) and rAAV-hTGF-β a human TGF beta 1 (hTGF-β) cDNA fragment (1.2 kb), both under the control of the cytomegalovirus immediate-early (CMV-IE) promoter [22, 23, 28, 31]. Conventional (not self-complementary) rAAV vectors were packaged using the 293 adenovirus-transformed embryonic kidney cell line. Helper functions were provided by Adenovirus 5 in combination with rep and cap functions of a pAd8 helper plasmid as previously described [28]. Purification, dialysis and titration of the vector preparations via real-time PCR were performed, averaging 10^10 transgene copies/ml with approximately 1/500 functional recombinant viral particles [22, 23, 28, 31].

Biochemical analyses

The aspirates were resuspended in a total volume of 100 µl of fresh DMEM and digested with papain (final concentration 75 µg/ml) at 60°C.

### Table 1

| Pathway               | Medium                                                                 |
|-----------------------|------------------------------------------------------------------------|
| Chondrogenesis        | 4.5 g/l DMEM high glucose, 100 U/ml penicillin, 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 and 10 ng/ml TGF-β3 |
| Osteogenesis          | StemPro Osteogenesis Differentiation Kit (Life Technologies, Darmstadt, Germany) |
| Adipogenesis          | StemPro Adipogenesis Differentiation Kit (Life Technologies) |

rAAV-mediated gene transfer

Bone marrow was aspirated from the distal femurs of patients undergoing total knee arthroplasty (~10 ml, n = 5). All patients included in the study provided informed consent and the procedures were in accordance with the Helsinki Declaration. The study was approved by the Ethics Committee of the Saarland Physicians Council (Application 06/08). Aspirates were immediately aliquoted in a volume of 100 µl per well in 96-well plates and transduced with 40 µl vector (i.e. 8 × 10^5 functional recombinant viral particles, MOI = 10 ± 3) [22, 23]. Samples were incubated for up to 21 days with various differentiation medium (Table 1), with careful weekly medium change to induce chondro-, osteo-, and adipogenic differentiation [22, 23]. Under continuous chondrogenic induction, mostly MSCs in the samples will commit towards the chondrocyte phenotype [23]. As we previously noted that the use of these media does not impede the effects of gene transfer via rAAV on the differentiation processes versus basal medium in aspirates [23], uninduced conditions were not further tested here.

Transgene expression

Transforming growth factor-β production was monitored by ELISA at the denoted time-points by absorbance measurements on a GENios spectrophotometer/fluorometer (Tecan, Gransaisheim, Germany) and by immunohistochemistry using a specific TGF-β antibody, a biotinylated secondary antibody, and dianinobenzidine as a chromogen (ABC method) [28, 31]. A control condition with omission of the primary antibody was included to check for secondary immunoglobulins. All sections were examined under light microscopy (Olympus BX45; Olympus, Hamburg, Germany).
Histological and immunohistochemical analyses

Aspirates were collected and fixed in 4% formalin with subsequent dehydration in graded alcohols, paraffin embedding and sectioning at 3 µm. Haematoxylin and eosin staining was performed to evaluate cellularity and toluidine blue and alizarin red staining for the detection of matrix proteoglycans and matrix mineralization respectively [22, 23]. The expression of type-II, -I and -X collagen and of SOX9 was evaluated by immunohistochemistry using specific primary antibodies, biotinylated secondary antibodies and the ABC method [22, 23]. Control conditions were included by omitting the primary antibodies. All sections were examined under light microscopy (Olympus BX45; Olympus).

Histomorphometry

Transgene expression was monitored by evaluating the percentage of TGF-β+ cells to the total number of cells on immunohistological sections [23]. Cell proliferation was evaluated by counting the total cells per standardized area on haematoxylin and eosin-stained sections [23]. The intensities of toluidine blue and alizarin red staining and those of type-II, -I and -X collagen and SOX9 immunostaining were monitored at the intensities of toluidine blue and alizarin red staining for the detection of matrix proteoglycans and matrix mineralization respectively [22, 23]. The expression of type-II, -I and -X collagen and of SOX9 was evaluated by immunohistochemistry using specific primary antibodies, biotinylated secondary antibodies and the ABC method [22, 23]. Control conditions were included by omitting the primary antibodies. All sections were examined under light microscopy (Olympus BX45; Olympus).

Real-time RT-PCR analyses

TRizol reagent (Ambion® Life Technologies, Thermo Scientific, Schwerte, Germany) and RNeasy Protect Mini Kit (Qiagen, Hilden, Germany) were used to extract total cellular RNA from all chondrogenically differentiated aspirates on day 21 post-transduction. The procedure included an on-column RNase-free DNase treatment (Qiagen) and extracted RNA was eluted in 30 µl of RNase-free water followed by reverse transcription using the 1st Strand cDNA Synthesis kit for RT-PCR (AMV; Roche Applied Science, Mannheim, Germany) with aliquots of 8 µl RNA eluate. The resulting cDNA products (±2 µl per sample) were finally amplified by real-time RT-PCR with Brilliant SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Waldbronn, Germany) on an Mx3000P QPCR operator system (Stratagene) under the following conditions: (95 °C, 10 min.), amplification by 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.). Primers for selected gene profiles are listed in Table 2 and applied at a final concentration of 150 nm. Controls consisting of water and non-reverse-transcribed mRNA were included and confirmation of the product specificities was done via melting curve analysis and agarose gel electrophoresis as previously described [23]. The MxPro QPCR Software (Stratagene) was used for measurements of the threshold cycle (Ct) value of each gene of interest and all values were normalized to GAPDH expression using the 2–DDCT method [23].

Statistical analyses

Each condition was performed in duplicate in two independent experiments for each patient. Data are expressed as mean ± S.D. of separate

| Table 2 Primers used for RT-PCR |
|---|
| Gene | Primer sequences (5’-3’) |
| SOX9 | ACACACAGCTCAGCTGACCTG GGGATTCTGTTGGTCCTCT |
| COL2A1 | GGAGTTTTCTCCTGCTCT GACCCGAAAGGTCTTACAGGA |
| ACAN | GAGATGGAGGTTGAGGTC AAGCTGGCTGGGCTCT |
| COL1A1 | GCAGTTCCCAAGCATTTCAT ACGGAGGGAAATGTTGTC |
| COL10A1 | CCAACTTGATGCCCACC AAATTTCACTTTTGGCAATGA |
| MMP13 | AATTTTCATTGTGAGATGATGAA CAAATAATTGTGAAAAAGGATG |
| ALP | TGGAGCCTTACAGGATGAACCATCA ACCTGTTGCTGAGTACCAGT |
| RUNX2 | CAGCGTCCACAGGATATTTC ACAATAATTGTGAAAAAGGATG |
| GAPDH | GAAGTGGAAAGGTCGAGTC GAAGATGGATGGATGGATG |

*Chondrogenic markers.  
†Osteogenic markers.  
‡Hypertrophic and terminal differentiation markers.  
§Housekeeping gene (control).  
SOX9: SRY (sex determining region Y)-box 9; COL2A1: type-II collagen α1; ACAN: aggrecan; COL1A1: type-I collagen α1; COL10A1: type-X collagen α1; MMP13: matrix metallo-proteinase 13; ALP: alkaline phosphatase; RUNX2: runt-related transcription factor 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
experiments. The t-test and Mann-Whitney rank-sum test were used where appropriate. Any P-value of less than 0.05 was considered statistically significant.

Results

Effective TGF-β overexpression via rAAV in chondrogenically induced human bone marrow aspirates

Human bone marrow aspirates were first transduced with the candidate rAAV-hTGF-β vector versus control (rAAV-lacZ) condition and cultivated for up to 21 days to test the ability of rAAV to promote the overexpression of the candidate TGF-β factor in conditions of chondrogenic induction. Transgene expression analyses revealed significantly higher immunoreactivity to TGF-β in the aspirates transduced with rAAV-hTGF-β compared with rAAV-lacZ (1553.2 ± 147.0 and 1056.9 ± 221.1 pixels/cell area after 21 days in the TGF-β-versus lacZ-treated samples, respectively, i.e. a 1.5-fold difference, P = 0.002) (Fig. 1). These results were supported by an estimation of the TGF-β production levels, revealing significantly higher values on days 7 and 21 (up to 2.9-fold difference, P ≤ 0.021) (Table 3).

Proliferative and chondrogenic effects of rAAV-mediated overexpression of TGF-β in chondrogenically induced human bone marrow aspirates

The aspirates were next transduced with rAAV-hTGF-β compared with rAAV-lacZ and induced towards chondrogenesis to evaluate the effects of TGF-β overexpression upon the proliferative activities and potential for chondrogenic differentiation in the samples. As we previously reported a lack of deleterious effects of rAAV gene transfer upon the potency of bone marrow aspirates [22], we did not further include a condition without vector treatment in this study.

A quantitative analysis by haematoxylin and eosin staining revealed significantly higher cell densities in the presence of rAAV-hTGF-β compared with rAAV-lacZ (402.0 ± 98.9 and 276.0 ± 66.2 pixels/cell area and 118.8 ± 27.1 and 47.2 ± 27.2 cells/mm² after 21 days in the TGF-β-versus lacZ-treated samples, respectively, i.e. an up to 2.5-fold difference, always P ≤ 0.004) (Fig. 2). These results were further confirmed via a biochemical assay to monitor the DNA contents in the aspirates, revealing significantly higher values with rAAV-hTGF-β compared with rAAV-lacZ (3.6 ± 2.0 and 0.7 ± 0.6 µg/mg total proteins after 21 days in the TGF-β-versus lacZ-treated samples, respectively, i.e. a 5.1-fold difference, P = 0.018) (Fig. 2).

To monitor the effects of TGF-β overexpression upon the chondrogenic events in the aspirates, the samples were further processed for the cartilage-specific proteoglycans, type-II collagen, and SOX9 markers by histological and immunohistochemical analyses. Significantly increased intensities were detected in the aspirates transduced

**Table 3** Production of TGF-β in chondrogenically induced human bone marrow aspirates via rAAV-mediated gene transfer

| Days post-transduction | rAAV-lacZ   | rAAV-hTGF-β |
|------------------------|-------------|-------------|
| 7                      | 178.5 (10.0)| 511.1 (116.9)* |
| 14                     | 49.4 (7.1)  | 112.5 (56.3) |
| 21                     | 5.8 (3.1)   | 16.1 (5.9)*  |

*Statistically significant compared with rAAV-lacZ.
Values are expressed as means pg/24 hrs/mg total proteins (S.D.).
with rAAV-hTGF-β compared with rAAV-lacZ for toluidine blue staining (1059.4 ± 284.1 and 545.1 ± 173.7 pixels/cell area after 21 days in the TGF-β- versus lacZ-treated samples, respectively, i.e. a 1.9-fold difference, \( P = 0.005 \)), anti-type-II collagen immunostaining (1151.5 ± 260.8 and 888.9 ± 85.8 pixels/cell area after 21 days in the TGF-β- versus lacZ-treated samples, respectively, i.e. a 1.3-fold difference, \( P = 0.037 \)), and anti-SOX9 immunostaining (1069.8 ± 76.8 and 938.9 ± 20.9 pixels/cell area after 21 days in the TGF-β- versus lacZ-treated samples, respectively, i.e. an up to 3.4-fold difference, always \( P \leq 0.047 \)) (Fig. 3). In addition, an analysis of the gene expression profiles in the aspirates by real-time RT-PCR revealed increases in the expression of ACAN (1.6-fold), COL2A1 (1.4-fold), and SOX9 (6.8-fold) after 21 days in the aspirates transduced with rAAV-hTGF-β compared with rAAV-lacZ (always \( P \leq 0.028 \)) (Fig. 5).

Hypertrophic/terminal differentiation was also monitored in the aspirates to evaluate the impact of TGF-β overexpression in the samples. An immunohistochemical analysis revealed significantly lower intensities in the aspirates transduced with rAAV-hTGF-β compared with rAAV-lacZ for alizarin red staining (734.9 ± 48.0 and 968.7 ± 192.6 pixels/cell area after 21 days in the TGF-β- versus lacZ-treated samples, respectively, i.e. a 1.3-fold difference, \( P = 0.016 \)), type-I collagen immunostaining (789.5 ± 64.7 and 921.2 ± 117.9 pixels/cell area after 21 days in the TGF-β- versus lacZ-treated samples, respectively, i.e. a 1.3-fold difference, always \( P \leq 0.047 \)) (Fig. 3). In addition, an analysis of the gene expression profiles in the aspirates by real-time RT-PCR revealed increases in the expression of ACAN (1.6-fold), COL2A1 (1.4-fold), and SOX9 (6.8-fold) after 21 days in the aspirates transduced with rAAV-hTGF-β compared with rAAV-lacZ (always \( P \leq 0.028 \)) (Fig. 5).
lacZ-treated samples, respectively, i.e. a 1.2-fold difference, \(P = 0.043\), and type-X collagen immunostaining \((777.4 \pm 136.1\) and \(992.3 \pm 116.3\) pixels/cell area after 21 days in the TGF-\(\beta\)- versus lacZ-treated samples, respectively, i.e. a 1.3-fold difference, \(P = 0.001\) (Fig. 4). A biochemical analysis performed to estimate the type-I and type-X collagen contents supported these findings, showing lower values in the presence of rAAV-hTGF-\(\beta\) \((8.0 \pm 0.1\) and \(10.4 \pm 0.9\) ng type-I collagen/mg total proteins and \(2.6 \pm 0.7\) and \(4.1 \pm 0.9\) ng type-X collagen/mg total proteins after 21 days in the TGF-\(\beta\)- versus lacZ-treated samples, respectively, up to a 1.6-fold difference, always \(P \leq 0.041\) (Fig. 4). Also, an analysis of the gene expression profiles in the aspirates monitored by real-time RT-PCR revealed decreases in the expression of type-I (4.3-fold) and type-X collagen (13.1-fold) after 21 days in the aspirates transduced with rAAV-hTGF-\(\beta\) compared with rAAV-lacZ (always \(P \leq 0.003\) (Fig. 5).

**Effects of rAAV-mediated overexpression of TGF-\(\beta\) in osteogenically and adipogenically induced human bone marrow aspirates**

The aspirates were also transduced with rAAV-hTGF-\(\beta\) compared with rAAV-lacZ and induced towards osteogenesis and adipogenesis to evaluate the influence of TGF-\(\beta\) overexpression on the potential for osteo-/adipogenic differentiation in the samples. While a trend towards enhanced adipogenic differentiation (Oil Red O staining) was seen in the presence of rAAV-hTGF-\(\beta\) compared with rAAV-lacZ \((4.13 \pm 0.36\) and \(2.04 \pm 0.37\) OD\(530\)nm after 21 days in the TGF-\(\beta\)-versus lacZ-treated samples, respectively, i.e. a twofold difference), osteogenic differentiation (ALP activity) was attenuated with TGF-\(\beta\) \((0.49 \pm 0.07\) and \(0.75 \pm 0.25\) OD\(530\)nm after 21 days in the TGF-\(\beta\)-versus lacZ-treated samples, respectively, i.e. a 1.5-fold difference), although statistical significance was not reached in the conditions applied here \((P = 0.076\) and \(P = 0.234\) respectively) (Fig. 6).

**Discussion**

Administration of genetically modified marrow concentrates to site of cartilage lesions via transfer of chondrogenic and/or chondroreparative factors using the clinically adapted rAAV vectors may provide convenient, single-step therapeutic approaches to enhance cartilage repair compared with the more complex and invasive implantation of isolated progenitor cells [6]. In this study, we examined the ability of rAAV to deliver a functional TGF-\(\beta\) gene cassette to human marrow concentrates in the light of the pleiotropic, chondrogenic properties of this growth factor [24–27] and with our previous findings showing that such a strategy was capable of stimulating such activities in isolated human MSCs [28].

We first observed that transduction with rAAV led to higher levels of TGF-\(\beta\) expression in the hTGF-\(\beta\)-treated concentrates...
compared with control (lacZ) treatment over the period of evaluation (21 days), in good agreement with our previous results in hMSCs [28]. The concentrations achieved via rAAV-hTGF-β gene transfer over time were in the range of those noted in hMSCs (~16.1 and ~24.1 pg rhTGF-β/mg total proteins on day 21 in the aspirates and in isolated cells respectively) [28]. Our findings further indicate that overexpression of TGF-β via rAAV led to increased levels of proliferative, biosynthetic and chondrogenic activities in the aspirates over time relative to control treatment, possibly because of the prolonged production of the growth factor permitted by stable rAAV gene transfer [32] and concordant with the effects of TGF-β when applied in a recombinant form [24–27] or used by us to target isolated hMSCs [28]. Of note, the levels of proliferation in the TGF-β-treated aspirates were higher than those noted with hMSCs (~3.6 μg versus ~26.4 ng DNA/mg total proteins, respectively, i.e. a 136-fold difference) [28], possibly because of the presence of other mitogenic factors in the aspirates and/or to effects from other marrow cell populations modified early on by rAAV while later on, under continuous chondrogenic activation mostly chondrogenically induced, rAAV-modified MSCs may control this process (paracrine versus autocrine effects). We thus anticipate that their depletion may prevent the effects of TGF-β in conditions of permanent chondrogenic activation over the current extended period of evaluation. Still, work would be needed to confirm this hypothesis by modifying aspirates devoid of the MSC subpopulation. In contrast, the levels of matrix synthesis were lower in the aspirates compared with those observed in isolated hMSCs (~2.7 versus ~9.5 ng type-II collagen/mg total proteins on day 21, respectively, i.e. a 3.5-fold difference; ~64.3 ng versus ~378.5 μg proteoglycans/mg total proteins, respectively, i.e. a 5.8 × 10^4-fold difference) [28], possibly because of a lower state of activation of chondroprogenitor cells in the aspirates compared with that acquired during cell expansion, to adverse effects from distinct, residual cells, and/or to prevalent mitogenic versus anabolic effects of TGF-β in asparites as noted in chondrocytes [33]. It cannot be excluded that the levels of matrix synthesis may still increase upon TGF-β treatment beyond the time-point selected here according to the relevant literature [19, 20, 23]. Such effects are most likely mediated by binding of TGF-β to its receptor (TβRII-TβRII complex with R-Smad cascade) and work is ongoing to test this hypothesis by performing receptor blocking studies [34]. Finally, we report that in the conditions tested here, application of the candidate rAAV-hTGF-β vector delayed hypertrophic and osteogenic differentiation in the aspirates, in association with reduced levels of MMP13 (marker of terminal differentiation), ALP (osteogénic marker) and RUNX2 (transcription factor controlling the osteoblastic expression of COL1, COL10 and MMP13). This is in striking contrast with our previous findings in isolated hMSCs, where the same vector stimulated instead these processes compared with control treatment [28]. Again, this might be the result of effects from other, beneficial factors present in the aspirates and/or of specific control pathways possibly activated in the presence of other marrow cell populations [35].

In conclusion, the results of this study show the value of providing therapeutic rAAV vectors to human bone marrow concentrates to enhance the chondrogenic differentiation processes for implantation in sites of cartilage injury. Work is ongoing to support the practicability of the approach by applying modified aspirates to focal defects [6, 19–21, 36, 37]. Also important, the cell subpopulations promoting the chondrogenic events in the aspirates are currently being characterized [7, 8, 38] to address the cell heterogeneity of the aspirates. Also, it remains to be seen whether gene transfer of TGF-β will be sufficient to achieve full repair in the lesions, and co-application of other candidates might be necessary, like for instance the cartilage oligomeric matrix protein, BMPs, IGF-I, basic fibroblast growth factor-2, SOX transcription factors, zinc-finger protein 145 (ZNF145), Indian hedgehog (Ihh) or human telomerase (hTERT) [22, 23, 39–47]. Combined gene transfer will be possible using rAAV as transduction with this class of vectors does not lead to gene transfer interference [48]. Overall, our results show the benefits of a direct modification of marrow aspirates via rAAV for the future treatment of cartilage defects.

Acknowledgements

This research was funded by grants from the German Osteoarthritis Foundation (Deutsche Arthrose-Hilfe e.V.). We thank R. J. Samulski (The Gene Therapy Center, University of North Carolina, Chapel Hill, NC), X. Xiao (The Gene Therapy Center, University of Pittsburgh, Pittsburgh, PA) and E. F. Tervoilliger (Division of Experimental Medicine, Harvard Institutes of Medicine and Beth Israel Deaconess Medical Center, Boston, MA) for providing genomic AAV-2 plasmid clones and the 293 cell line.

Conflicts of interest

The authors confirm that there are no conflicts of interest.
References

1. Buckwalter JA. Articular cartilage: injuries and potential for healing. J Orthop Sports Phys Ther. 1998; 28: 192–202.
2. Britberg M, Lindahl A, Nilsson A, et al. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med. 1994; 331: 889–95.
3. Knutsen G, Engebretsen L, Ludvigsen TC, et al. Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. J Bone Joint Surg Am. 2004; 86-A: 455–64.
4. Madry H, Grun UW, Knutsen G. Cartilage repair and joint preservation: medical and surgical treatment options. Disch Arztebl Int. 2011; 108: 669–77.
5. Dewan AK, Gibson MA, Elisseoft JH, et al. Evolution of autologous chondrocyte repair and comparison to other cartilage repair techniques. Biomed Res Int. 2014; 2014: 1274691–92.
6. Orth P, Rey-Rico A, Venkatesan JK, et al. Current perspectives in stem cell research for knee cartilage repair. Stem Cells Cloning. 2014; 7: 1–17.
7. Lennon DP, Haynesworth SE, Arm DM, et al. Dilution of human mesenchymal stem cells with dermal fibroblasts and the effects on in vitro and in vivo osteochondrogenesis. Dev Dyn. 2000; 219: 50–62.
8. Anam K, Davis TA. Comparative analysis of gene transcripts for cell signaling receptors in bone marrow-derived hematopoietic stem/progenitor cell and mesenchymal stromal cell populations. Stem Cell Res Ther. 2013; 4: 112–24.
9. Wakiyama T, Mitsuoka T, Nakamura N, et al. Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: two case reports. Cell Transplant. 2004; 13: 595–600.
10. Slynarski K, Deszczyzny J, Karpiński J. Fresh bone marrow and peristeum transplantation for cartilage defects of the knee. Transplant Proc. 2006; 38: 318–9.
11. Gigante A, Cecconi S, Calcagno S, et al. Arthroscopic knee cartilage repair with covered microfracture and bone marrow concentrate. Arthrosc Tech. 2012; 1: e175–80.
12. Enea D, Cecconi S, Calcagno S, et al. Single-stage cartilage repair in the knee with microfracture covered with a resorbable polymer-based matrix and autologous bone marrow concentrate. Knee. 2013; 20: 562–9.
13. Orozco L, Munar A, Soler R, et al. Treatment of knee osteoarthritis with autologous mesenchymal stem cells: a pilot study. Transplantation. 2013; 95: 1535–41.
14. Skowronska J, Skowronski R, Rutka M. Large cartilage lesions of the knee treated with bone marrow concentrate and collagen membrane–results. Ortop Traumatol Rehabil. 2013; 15: 69–76.
15. Kim JD, Lee GW, Jung GH, et al. Clinical outcome of autologous bone marrow aspirate concentrate (BMAC) injection in degenerative arthritis of the knee. Eur J Orthop Surg Traumatol. 2014; 24: 1505–11.
16. Cucchiari M, Madry H, Guilk F, et al. A vision on the future of articular cartilage repair. Eur Cell Mater. 2014; 27: 12–6.
17. Johnstone B, Alini M, Cucchiari M, et al. Tissue engineering for articular cartilage repair—the state of the art. Eur Cell Mater. 2013; 25: 248–67.
18. Frisch J, Venkatesan JK, Rey-Rico A, et al. Current progress in stem cell-based gene therapy for articular cartilage repair. Curr Stem Cell Res Ther. 2015; 10: 121–31.
19. Pascher A, Palmer GD, Steinert A, et al. Gene delivery to cartilage defects using coagulated bone marrow aspirate. Gene Ther. 2004; 11: 133–41.
20. Ivkovic A, Pascher A, Hudez D, et al. Articular cartilage repair by genetically modified bone marrow aspirate in sheep. Gene Ther. 2010; 17: 779–89.
21. Sieker JT, Kunz M, Weissenberger M, et al. Direct bone morphogenetic protein 2 and Indian hedgehog gene transfer for articular cartilage repair using bone marrow coagulates. Osteoarthritis Cartilage. 2015; 23: 433–42.
22. Rey-Rico A, Frisch J, Venkatesan JK, et al. Determination of effective rAAV-mediated gene transfer conditions to support chondrogenic differentiation processes in human primary bone marrow aspirates. Gene Ther. 2015; 22: 50–7.
23. Frisch J, Rey-Rico A, Venkatesan JK, et al. Chondrogenic differentiation processes in human bone marrow aspirates upon rAAV-mediated gene transfer and overexpression of the insulin-like growth factor I (IGF-I) overexpression for the long-term reconstruction of human osteoarthritic cartilage by modulation of the IGF-1 axis. Mol Med. 2012; 18: 346–58.
24. Jonitz A, Lochner K, Tischer T, et al. TGF-beta and IGF-1 influence the re-differentiation capacity of human chondrocytes in 3D pellet cultures in relation to different oxygen concentrations. Int J Mol Med. 2012; 30: 666–72.
25. Longobardi L, O’Rear L, Aakula S, et al. Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-beta signaling. J Bone Miner Res. 2006; 21: 626–36.
26. Pinttenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284: 143–7.
27. Barry F, Boynton RE, Liu B, et al. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res. 2001; 268: 189–200.
28. Frisch J, Venkatesan JK, Rey-Rico A, et al. Determination of the chondrogenic differentiation processes in human bone marrow–derived mesenchymal stem cells modified to overexpress transforming growth factor-beta via recombinant adenovirus vectors. Hum Gene Ther. 2014; 25: 1050–60.
29. Samulski RJ, Chang LS, Shenk T. A recombinant plasmid from which an infectious adenovirus genome can be excised in vitro and its use to study viral replication. J Virol. 1987; 61: 3096–101.
30. Samulski RJ, Chang LS, Shenk T. Helper–free stocks of recombinant adenovirus: normal integration does not require viral gene expression. J Virol. 1989; 63: 3822–8.
31. Venkatesan JK, Rey-Rico A, Schmitt G, et al. rAAV-mediated overexpression of TGF-beta stably restructures human osteoarthritic articular cartilage in situ. J Transl Med. 2013; 11: 211–24.
32. Weimer A, Madry H, Venkatesan JK, et al. Benefits of recombinant adenovirus vector (rAAV)-mediated insulin-like growth factor I (IGF-I) overexpression for the long-term reconstruction of human osteoarthritic cartilage by modulation of the IGF-1 axis. Mol Med. 2012; 18: 346–58.
33. Jonitz A, Lochner K, Tischer T, et al. TGF-beta and IGF-1 influence the re-differentiation capacity of human chondrocytes in 3D pellet cultures in relation to different oxygen concentrations. Int J Mol Med. 2012; 30: 666–72.
34. Longobardi L, O’Rear L, Aakula S, et al. Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-beta signaling. J Bone Miner Res. 2006; 21: 626–36.
35. Yang X, Chen L, Xu X, et al. TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol. 2001; 153: 35–46.
36. Cucchiari M, Orth P, Madry H. Direct rAAV SOX9 administration for durable articular cartilage repair with delayed terminal differentiation and hypertrophy in vivo. J Mol Med. 2013; 91: 625–36.

© 2016 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.
Cucchiarini M, Madry H. Overexpression of human IGF-I via direct rAAV-mediated gene transfer improves the early repair of articular cartilage defects in vivo. Gene Ther. 2014; 21: 811–9.

Jones EA, Kinsey SE, English A, et al. Isolation and characterization of bone marrow multipotent mesenchymal progenitor cells. Arthritis Rheum. 2002; 46: 3349–60.

Ikeda T, Kamekura S, Mabuchi A, et al. The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis Rheum. 2004; 50: 3561–73.

Bocker W, Yin Z, Drosse I, et al. Introducing a single-cell-derived human mesenchymal stem cell line expressing hTERT after lentiviral gene transfer. J Cell Mol Med. 2008; 12: 1347–59.

Cucchiarini M, Ekici M, Schetting S, et al. Metabolic activities and chondrogenic differentiation of human mesenchymal stem cells following recombinant adeno-associated virus-mediated gene transfer and overexpression of fibroblast growth factor 2. Tissue Eng Part A. 2011; 17: 1921–33.

Kim HJ, Im GI. Electroporation-mediated transfer of SOX trio genes (SOX-5, SOX-6, and SOX-9) to enhance the chondrogenesis of mesenchymal stem cells. Stem Cells Dev. 2011; 20: 2103–14.

Liu TM, Guo XM, Tan HS, et al. Zinc-finger protein 145, acting as an upstream regulator of SOX9, improves the differentiation potential of human mesenchymal stem cells for cartilage regeneration and repair. Arthritis Rheum. 2011; 63: 2711–20.

Haleem-Smith H, Calderon R, Song Y, et al. Cartilage oligomeric matrix protein enhances matrix assembly during chondrogenesis of human mesenchymal stem cells. J Cell Biochem. 2012; 113: 1245–52.

Steinert AF, Weissenberger M, Kunz M, et al. Indian hedgehog gene transfer is a chondrogenic inducer of human mesenchymal stem cells. Arthritis Res Ther. 2012; 14: R168–80.

Venkatesan JK, Ekici M, Madry H, et al. SOX9 gene transfer via safe, stable, replication-defective recombinant adeno-associated virus vectors as a novel, powerful tool to enhance the chondrogenic potential of human mesenchymal stem cells. Stem Cell Res Ther. 2012; 3: 22–36.

Neumann AJ, Alini M, Archer CW, et al. Chondrogenesis of human bone marrow-derived mesenchymal stem cells is modulated by complex mechanical stimulation and adenoviral-mediated overexpression of bone morphogenetic protein 2. Tissue Eng Part A. 2013; 19: 1285–94.

Cucchiarini M, Terwilliger EF, Kohn D, et al. Remodelling of human osteoarthritic cartilage by FGF-2, alone or combined with Sox9 via rAAV gene transfer. J Cell Mol Med. 2009; 13: 2476–88.