Peripheral blood aspirates overexpressing IGF-I via rAAV gene transfer undergo enhanced chondrogenic differentiation processes

Janina Frisch a, Patrick Orth a, b, Ana Rey-Rico a, Jagadeesh Kumar Venkatesan a, Gertrud Schmitt a, Henning Madry a, b, Dieter Kohn 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: January 4, 2017; Accepted: March 9, 2017

Abstract
Implantation of peripheral blood aspirates induced towards chondrogenic differentiation upon genetic modification in sites of articular cartilage injury may represent a powerful strategy to enhance cartilage repair. Such a single-step approach may be less invasive than procedures based on the use of isolated or concentrated MSCs, simplifying translational protocols in patients. In this study, we provide evidence showing the feasibility of overexpressing the mitogenic and pro-anabolic insulin-like growth factor I (IGF-I) in human peripheral blood aspirates via rAAV-mediated gene transfer, leading to enhanced proliferative and chondrogenic differentiation (proteoglycans, type-II collagen, SOX9) activities in the samples relative to control (reporter rAAV-lacZ) treatment over extended periods of time (at least 21 days, the longest time-point evaluated). Interestingly, IGF-I gene transfer also triggered hypertrophic, osteo- and adipogenic differentiation processes in the aspirates, suggesting that careful regulation of IGF-I expression may be necessary to contain these events in vivo. Still, the current results demonstrate the potential of targeting human peripheral blood aspirates via therapeutic rAAV transduction as a novel, convenient tool to treat articular cartilage injuries.

Keywords: cartilage repair ● gene therapy ● peripheral blood ● rAAV vectors ● IGF-I

Introduction
Damaged adult hyaline articular cartilage, the tissue that is essential for weight absorption and smooth gliding of the articulating surfaces in diarthroidal joints, does not naturally undergo competent repair processes in the absence of blood vessels and lymphatic drainage that may provide regenerative progenitor cells in sites of injury [1]. Lesions such as traumatic focal defects or occurring in osteoarthritis (OA) are infiltrated by cells migrating from the synovial membrane that fail to produce high-quality repair tissue, leading to progression of the injury over time [2].

Current therapeutic options include marrow stimulation techniques to give access to the subchondral bone marrow to the lesions [2] as this compartment contains chondroreparative mesenchymal stem cells (BM-MSCs) that may enhance the healing of the injured tissue [3,4]. Transplantation of bone marrow aspirates or concentrates has been also attempted to further enhance the processes of cartilage repair [5–10] compared with the more tedious application of isolated and expanded MSCs [2, 11–13] or of articular chondrocytes [14, 15], leading to improved outcome in the patients. However, close evaluations revealed that the repair tissue displayed a relatively poor structural organization, integrity and biomechanical function that did not match the original hyaline cartilage. The use of peripheral blood may provide workable, less invasive translational procedures as this compartment also contains MSCs (PB-MSCs) with the same potency for chondrogenic differentiation as that of BM-MSCs [16–18], including in vivo when applied as isolated populations [9, 19–21]. As such setups led again to an incomplete reconstitution of the natural cartilage structure [9, 19–21], new strategies may need to be considered to generate improved therapeutic regimens. In particular, genetic modification of such samples may be a powerful tool to stimulate the chondroreparative processes of PB-MSCs [22]. Among the various gene transfer systems available to achieve this goal, vehicles based on the human adeno-associated virus (AAV) offer a number of benefits for translational research as recombinant AAV (rAAV) vectors are much less immunogenic and toxic and more efficient than nonviral, adenoviral and retro-/lentiviral vectors due to the
absence of viral sequences in the recombinant genome and to the long-term maintenance of the rAAV transgenes under episomal forms [23, 24].

While genetic modification of human bone marrow aspirates via rAAV vectors has been reported in a variety of studies using growth (transforming growth factor β, i.e. TGF-β), basic fibroblast growth factor, i.e. FGF-2, IGF-I) [4, 25, 26] and transcription factors (SOX9) [27] for chondrogenic purposes, thus far there is little evidence on the possibility of targeting human peripheral blood aspirates with this vector class. We were actually the first group to provide evidence that overexpression of TGF-β via rAAV significantly enhanced the chondrogenic differentiation processes in such samples [1]. Yet, as the treatment also stimulated hypertrophy and osteogenic differentiation events, new avenues of research to identify other candidate genes are needed for a safe translation of the strategy in future in vivo settings.

In the light, the mitogenic and pro-anabolic IGF-I factor may be a good alternative as it displays chondroreparative activities when needed for a safe translation of the strategy in future in vivo settings.

**Materials and methods**

**Reagents**

Recombinant TGF-β (rTGF-β) was purchased at Peprotech (Hamburg, Germany), dimethylmethyleneblue (DMMB) at Serva (Heidelberg, Germany) and dianinobenzidine (DAB) at Sigma-Aldrich (Munich, Germany). The anti-IGF-I (AF-291-NA) antibody was from R&D Systems (Wiesbaden, Germany). The anti-SOX9 (C-20) antibody from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-IGF-I (AF-291-NA) antibody was from R&D Systems (Minneapolis, USA) or adipogenesis (StemPro Adipogenesis Differentiation Kit; Life Technologies, Darmstadt, Germany) or diaminobenzidine (DAB) at Sigma-Aldrich (Munich, Germany), dimethylmethyleneblue (DMMB) at Serva (Heidelberg, Germany). The hIGF-I Quantikine ELISA was purchased at R&D Systems (Minneapolis, USA). The anti-IGF-I (AF-291-NA) antibody was from R&D Systems (Minneapolis, USA). The ABC kit were from Vector Laboratories (Alexis Deutschland GmbH, Grünberg, Germany). The anti-type-II collagen (II-II6B3) antibody from Abcam (Cambridge, UK). The anti-IGF-I (AF-291-NA) antibody was from R&D Systems (Minneapolis, USA). The anti-IGF-I (AF-291-NA) antibody was from R&D Systems (Minneapolis, USA).

Peripheral blood aspirates were immediately aliquoted in 96-well plates (100 µl/well) after collection and directly mixed with the rAAV vectors using vector doses known to allow for optimal transduction efficiencies (40 µl, i.e. 8 x 10^5 functional recombinant viral particles, MOI = 10 ± 3) [1]. After a short incubation time (1–2 min.), 50 µl of supplement-free DMEM was added, and the mixture was further incubated for 90 min. at 37°C and 5% CO2 [1]. Finally, 60 µl of specific media was mixed with the transduced samples to induce chondrogenesis (4.5 g/l DMEM high glucose, 100 µU/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^{-5} M dexamethasone and 10 ng/ml TGF-β3), osteogenesis (StemPro Osteogenesis Differentiation Kit; Life Technologies, Darmstadt, Germany) or adipogenesis (StemPro Adipogenesis Differentiation Kit; Life Technologies) [1]. Careful medium change was performed once per week for up to 21 days of incubation [1]. All aspirates formed early on clots that remained intact over the whole period of time.

**Transgene expression**

Immunohistochemical staining was performed to detect transgene (IGF-I) expression using a specific primary antibody, a biotinylated secondary antibody and the ABC kit method using DAB as the chromogen [1]. Sections were recorded under light microscopy (Olympus BC45; Olympus, Hamburg, Germany) at 20× magnification for histomorphometric analyses [1]. IGF-I secretion was monitored by ELISA using cell culture supernatants at the denoted time-points and 24 hrs after medium change to conditioned medium [1] with a GENios spectrophotometer (Tecan, Crailsheim, Germany).

**Biochemical analyses**

Aspirates were collected in a volume of 100 µl of fresh, supplement-free DMEM, followed by papain digestion (final concentration: 75 µg/ml; 60 min. at 60°C) [1]. The amounts of total proteins were detected using the Pierce BCA Protein Assay Kit (Pierce,
ThermoFisher Scientific, Schwerte, Germany) for normalization of biochemical measurements [1]. Evaluation of the DNA content was carried out using the Hoechst 23358 assay and the proteoglycan contents by binding to the DMMB dye [1]. Type-II, -I and -X collagen contents were monitored by respective ELISA according to the manufacturer’s protocols [1]. Osteogenesis was assessed by mixing osteogenically induced aspirates with an equal volume of specific substrate buffer (4-nitrophenyl phosphate—pNPP—mixed 1:1 with 4.8% 2-amino-2-methyl-1-propanol—2-AMP) to analyse ALP activity by absorbance measurements [25]. Adipogenesis was evaluated via Oil Red O staining as previously described [25]. Briefly, adipogenically induced aspirates were mixed with 150 µl of staining solution (three volumes of Oil Red O 0.3% in 2-propanol and two volumes of H₂O), followed by incubation for 15 min. at room temperature and final dissolution in 100% 2-propanol for absorbance measurements. Evaluations were performed on a GENios spectrophotometer/fluorometer (Tecan).

Histological and immunohistochemical analyses

The samples were fixed in 4% formalin and embedded in paraffin after dehydration in graded alcohols, followed by sectioning at 3 µm to be placed on histological slides [1]. Haematoxylin and eosin staining (H&E) was carried out to evaluate cellularity [1]. The deposition of proteoglycan and matrix mineralization were visualized via toluidine blue and alizarin red staining respectively [1]. Immunohistochemical analyses were performed to detect the expression of type-II, -I and -X collagen as well as SOX9 using specific primary antibodies, biotinylated secondary antibodies and the ABC method with DAB as the chromogen [1]. Stained sections were recorded under light microscopy (Olympus BX45) at 20× magnification for histomorphometric analyses [1].

Histomorphometry

Staining intensities on histological (H&E, toluidine blue, alizarin red) and immunohistochemical (IGF-I, type-II, -I and -X collagen, SOX9) sections were analysed by measurement of pixels per standardized area (pixels/mm²) [1]. Images at 20× magnification were converted to greyscale mode, and inverted, background signal was adapted for comparable range and the mean grey value per total area covered with cells was detected [1]. All data were collected at three random standardized sites or using 10 serial histological and immunohistochemical sections for each parameter, test and replicate condition using the CellSens Standard programme (Olympus) and Adobe Photoshop (Adobe Systems, Unterschleissheim, Germany) [1].

Real-time RT-PCR analyses

Aspirates collected in a total volume of 100 µl fresh, supplement-free medium, were mixed with 6 volumes of TRIzol reagent (Ambion® Life Technologies) and 3 volumes of chloroform, followed by density gradient centrifugation [1]. The nucleic acid containing clear phase was further used to perform RNA isolation with the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany), including an on-column RNase-free DNase treatment (Qiagen) and elution in 30 µl of RNase-free water [1]. Small aliquots (8 µl) were used for reverse transcription using the 1st Strand cDNA Synthesis kit for RT-PCR (AMV; Roche Applied Science), followed by amplification via real-time RT-PCR with 2 µl of the newly synthesized cDNA using the Brilliant SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Waldbronn, Germany) and a Mx3000P QPCR operator system (Stratagene) [1]. The following cycle conditions were used: (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 were purchased at Invitrogen (Darmstadt, Germany) applied at a final concentration of 150 nm as follows: aggrecan (ACAN) (chondrogenic marker) (forward 5’-GAGATGGAGGTTGAGGTTC-3’; reverse 5’-AGCTGCTGCAGGCCTC-3’), type-II collagen (COL2A1) (chondrogenic marker) (forward 5’-GGACCTTTCTCCCTCTC-3’; reverse 5’-GACCCGAGGTCCTACAGGA-3’), SOX9 (chondrogenic marker) (forward 5’-ACACACAGCCTACCTGCTGACC-3’; reverse 5’-GGGAATTCCTGTTGCTC-3’), type-I collagen (COL1A1) (osteogenic marker) (forward 5’-ACCTGTCCTGTGAA GTTGGTC-3’; reverse 5’-ACGAGGAAAGCCTCTCTC-3’), type-X collagen (COL10A1) (marker of hypertrophy) (forward 5’-CCTCTTGTTAGTGGCAACCC-3’; reverse 5’-GATGCAAGCAAGCCCTTCCCAAC-3’), matrix metalloproteinase 13 (MMP13) (osteogenic marker) (forward 5’-TGAGGAACCTGAAAGCT CAAACCAA-3’; reverse 5’-ATCTGGTGTGTCGTTCTACAGC-3’), alkaline phosphatase (ALP) (osteogenic marker) (forward 5’-GGGTCCTGTTGACGAGCT-3’; reverse 5’-GGAGATTTCATTGTATGAA AAGGGATGC-3’), runt-related transcription factor 2 (RUNX2) (osteogenic marker) (forward 5’-GAATCCCAAAGCTACTCTC-3’; reverse 5’-GACTCTGGTCTGGGAAGAAGA-3’), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene and internal control) (forward 5’-GAAGATGCTGCGATGAGTGTTG-3’; reverse 5’-AAAGATGCGATGAGTGTTG-3’).

Statistical analysis

Two independent experiments were performed for each patient and condition to generate duplicates. All samples were tested for each evaluation. Data are represented as mean ± standard deviation (S.D.), and the t-test with normal distribution of data was used where appropriate, considering P ≤ 0.05 for statistically significant.

Results

Successful rAAV-mediated gene transfer and overexpression of IGF-I in chondrogenically induced human peripheral blood aspirates

Human peripheral blood aspirates were first transduced with the candidate rAAV-hIGF-I vector in order to examine the possibility of overexpressing the growth factor in the samples over time in conditions of continuous chondrogenic induction relative to control (no vector treatment, rAAV-lacZ gene transfer) conditions.

An immunohistochemical analysis revealed significantly higher IGF-I staining intensities in aspirates transduced with rAAV-hIGF-I...
compared with the control conditions (up to 1.2-fold difference on day 21, \( P \leq 0.05 \); Fig. 1A and B). An evaluation of the IGF-I production levels in the samples by ELISA further showed significantly higher amounts of growth factor expression with rAAV-hIGF-I relative to the control conditions at any time-point of the analysis (up to 1.7-fold difference, \( P \leq 0.05 \)), while no differences were noted between rAAV-lacZ and a lack of vector treatment (\( P \geq 0.050 \); Fig. 1C).

**Fig. 1** Transgene (IGF-I) expression analyses in rAAV-modified, chondrogenically induced human peripheral blood samples. Aspirates (\( n = 4 \)) were transduced with rAAV-lacZ or rAAV-hIGF-I (40 \( \mu l \) each vector) or left untreated and kept for up to 21 days in chondrogenic medium as described in the Materials and methods and analysed to monitor IGF-I transgene expression via immunohistochemistry (A) (magnification x20; left insets at magnification x40; right inserts at magnification x40 show sections without primary antibody; all representative data) with corresponding histomorphometric analyses (B) and by ELISA (C). Statistically significant compared with no vector treatment and rAAV-lacZ.

Enhanced proliferative and chondrogenic activities in chondrogenically induced human peripheral blood aspirates upon rAAV-mediated IGF-I overexpression

Human peripheral blood aspirates were then transduced with rAAV-hIGF-I to test the potential effects of IGF-I overexpression on the
proliferative and chondrogenic activities in the samples under continuous chondrogenic induction versus control (rAAV-lacZ) transduction. As we previously reported that rAAV-mediated gene transfer has no deleterious effects on the multipotency of such aspirates [1] and in the light of our results above on transgene expression showing no statistically significant difference between the two control aspirate types.

**Fig. 2** Proliferative activities in rAAV-modified, chondrogenically induced human peripheral blood aspirates. Aspirates (n = 4) were transduced as described in Fig. 1 and processed after 21 days for H&E staining (A) (magnification x20; insets at magnification x40; all representative data) with corresponding histomorphometric analyses (B) and to measure the DNA contents (C) as described in the Materials and methods. *Statistically significant compared with rAAV-lacZ.

**Fig. 3** Chondrogenic marker expression in rAAV-modified, chondrogenically induced human peripheral blood samples. Aspirates (n = 4) were transduced as described in Fig. 1 and processed after 21 days for toluidine blue staining with histomorphometric analyses and to measure the proteoglycan contents (A) (magnification x20; insets at magnification x40; all representative data) for type-II collagen immunostaining with histomorphometric analyses and to measure the type-II collagen contents (B) (magnification x20; left insets at magnification x40; right insets at magnification x40 show sections without primary antibody; all representative data) and for SOX9 immunostaining with histomorphometric analyses (C) (magnification x20; left insets at magnification x40; right insets at magnification x40 show sections without primary antibody; all representative data) as described in the Materials and methods. *Statistically significant compared with rAAV-lacZ.
Significantly increased cellularity was observed when applying rAAV-hIGF-I to the aspirates compared with rAAV-lacZ as noted on H&E-stained histological sections (up to 1.1-fold difference on day 21, \( P \leq 0.05 \); Fig. 2A and B). Similar results were noted when analysing the DNA contents in the transduced aspirates (1.5-fold increases with rAAV-hIGF-I relative to rAAV-lacZ on day 21, \( P \leq 0.05 \); Fig. 2C).

Enhanced levels of chondrogenic marker expression were also achieved in the aspirates upon overexpression of IGF-I compared with control (lacZ) treatment as seen by significantly increased staining and immunostaining intensities of toluidine blue, type-II collagen and SOX9 (always 1.1-fold difference on day 21, \( P \leq 0.05 \); Fig. 3A–C), a finding confirmed when estimating the proteoglycan and type-II collagen contents in the samples (up to 1.5-fold difference on day 21, \( P \leq 0.05 \); Fig. 3A and B). Furthermore, a real-time RT-PCR analysis revealed higher gene expression profiles for aggrecan (ACAN), COL2A1 and SOX9 in rAAV-hIGF-I-treated aspirates versus rAAV-lacZ (2.0-, 3.2- and 1.9-fold difference on day 21 respectively, \( P \leq 0.05 \); Fig. 4).

Enhanced levels of matrix calcification were achieved in the aspirates upon overexpression of IGF-I compared with control (lacZ) treatment as seen by significantly increased alizarin red staining (1.2-fold difference on day 21, \( P \leq 0.05 \); Fig. 5A). Significantly increased expression of type-I and -X collagen was also noted by immunohistochemical analyses in the presence of rAAV-hIGF-I relative to rAAV-lacZ (up to 1.2-fold difference on day 21, \( P \leq 0.05 \); Fig. 5B and C), a finding corroborated by an estimation of the type-I and -X collagen contents in the samples (up to 1.5-fold difference on day 21, \( P \leq 0.05 \); Fig. 5B and C). A real-time RT-PCR analysis finally demonstrated higher gene expression profiles for COL1A1, COL10A1 and for the transcription factor RUNX2, with GAPDH serving as a housekeeping gene and internal control for normalization (primers listed in the Materials and methods section).
Effects of rAAV-mediated IGF-I overexpression on the osteogenic and adipogenic differentiation processes in osteogenically and adipogenically induced human peripheral blood aspirates

Human peripheral blood aspirates were finally transduced with rAAV-hIGF-I versus rAAV-lacZ to test the potential effects of IGF-I overexpression on the osteogenic and adipogenic differentiation processes in the samples under continuous osteogenic or adipogenic induction.

Application of the IGF-I vector led to higher osteogenic and adipogenic activities in the aspirates compared with control (lacZ) treatment as noted by an estimation of the ALP and Oil Red O activities respectively (always 1.5-fold difference on day 21, P ≤ 0.05; Fig. 4).

Discussion

Direct modification of peripheral blood aspirates using the clinically relevant, highly effective rAAV gene vectors is a promising strategy to enhance the chondrogenic differentiation of PB-MSCs for further convenient therapeutic application in articular cartilage lesions [1]. In the light of our previous work showing the chondroreparative potency of rAAV IGF-I gene transfer in concentrated BM-MSCs [25], the approach developed here was to test whether this active construct was capable of enhancing the chondrogenic activities in freshly isolated peripheral blood aspirates from patients as the ultimate goal for single-step, minimal invasive therapy of cartilage injury.

The data first indicate that effective, significant overexpression of IGF-I was achieved in human peripheral blood aspirates upon rAAV gene transfer relative to the control (lacZ) treatment for up to 21 days, the longest time-point evaluated, concordant with our findings in human bone marrow concentrates [25]. Interestingly, while early on the levels of IGF-I production were higher in the IGF-I-treated peripheral blood aspirates (~150 versus 97 pg/mg total proteins/24 hrs in bone marrow samples, i.e. an ~1.5-fold difference, P ≤ 0.05), they decreased over time and became inferior to those noted in the bone marrow (~47 versus 106 pg/mg total proteins/24 hrs, i.e. an ~2.3-fold difference, P ≤ 0.05) [25]. This might be due to dissimilar biochemical (paracrine factors) and cellular microenvironments in the samples (different representation of MSCs: <0.0002% MSCs in the blood versus ~1% in the marrow; distinct populations of fibroblasts and hematopoietic cells) [16–18, 34–38], to a variability of permissivity to rAAV gene transfer and intracellular transgene processing in the samples [39] and/or to different clonal expansion of IGF-I-transduced populations in the aspirates [1, 25]. Work is ongoing to appreciate such discrepancies between samples by comparing patient-matched blood and marrow concentrates and to characterize the cell subpopulations amenable to rAAV gene transfer in chondrogenically induced the peripheral blood aspirates by immunophenotyping [1]. In the light of our previous findings in bone marrow aspirates [25], it is probable that in conditions of continuous chondrogenic induction, mostly MSCs capable of competently committing towards the chondrocyte phenotype in such an environment [34, 38] may contribute to the chondrogenic differentiation processes.

We next show that rAAV-mediated IGF-I overexpression enhanced the proliferative and chondrogenic activities in the peripheral blood aspirates over time (21 days) and relative to the control treatment, again in good agreement with our findings in human bone marrow concentrates [25] and with the properties of the growth factor [40]. Of note, the proliferative indices in the IGF-I-treated peripheral blood aspirates were lower than those achieved in the bone marrow (~32 versus 2700 ng DNA/mg total proteins, i.e. an ~84-fold difference, P ≤ 0.05) [25], possibly due to the differences in IGF-I production levels over time between the samples, to distinct levels of IGF-I receptor membrane expression in cells forming the aspirates, and/or to the lower frequency of chondrogenically competent MSCs in the blood relative to the bone marrow that may mostly be the subpopulation undergoing cell division over time in conditions of continuous chondrogenic stimulation [1]. Furthermore, the levels of chondrogenic activities in the IGF-I-treated peripheral blood aspirates were also inferior to those noted in the bone marrow (~20 versus 40 μg proteoglycans/mg total proteins and ~0.13 versus 0.30 ng type-II collagen/mg total proteins, i.e. an ~2- to 2.3-fold difference, P ≤ 0.05) [25], again possibly reflecting the lower levels of IGF-I produced via rAAV in the blood samples, the lesser representation of MSCs in these samples that may mostly be prone to chondrogenic commitment under continuous specific stimulation [1] and/or to discrepancies in the state of the osteogenic marker alkaline phosphatase (ALP) in rAAV-hIGF-I-treated aspirates versus rAAV-lacZ (2.2–2.4- and 1.9-fold difference on day 21, respectively, P ≤ 0.05; Fig. 4), concomitantly with increased expression of the marker of terminal differentiation MMP13 and of the osteogenic transcription factor RUNX2 (3.0- and 2.1-fold increased expression of the marker of terminal differentiation MMP13, respectively as described in the Materials and methods.

![Fig. 6 Osteogenic and adipogenic differentiation processes in rAAV-modified, osteogenically and adipogenically induced human peripheral blood aspirates. Aspirates (n = 4) were transduced as described in Figure 1, and the osteogenic and adipogenic differentiation pathways were assessed after 21 days by analysis of the ALP and Oil Red O activities, respectively as described in the Materials and methods. *Statistically significant compared with rAAV-lacZ.](image-url)
activation of these cells over time in the blood aspirates in their particular microenvironment. For comparison, similar levels of proliferation and chondrogenic activities were reported when applying a rAAV TGF-β construct to human blood aspirates using identical conditions of chondrogenic induction [1].

We finally demonstrate that gene transfer with rAAV IGF-I also promoted hypertrophic and osteogenic differentiation in peripheral blood aspirates over time and relative to the control treatment, corroborating our findings in human bone marrow concentrates [25] and with the properties of the growth factor [41]. Strikingly, the extent of such events was lower in the IGF-I-treated peripheral blood aspirates compared with the bone marrow (~80 versus 300 pg type-I collagen/mg total proteins and ~170 versus 400 pg type-X collagen/mg total proteins, i.e. an ~2.4- to 3.8-fold difference, P ≤ 0.05) [25], again possibly resulting from lower levels of IGF-I production via rAAV in the blood or to a more favourable microenvironment in this compartment that may allow to better contain such undesirable processes. Of further note, the levels of hypertrophic and osteogenic differentiation achieved in the human peripheral blood aspirates here with rAAV IGF-I were inferior to those seen when using an rAAV TGF-β construct in similar conditions of chondrogenic induction (~170 versus 2600 pg type-X collagen/mg total proteins, i.e. an ~15.3-fold difference, P ≤ 0.05) [1].

In summary, the present study shows the value of modifying peripheral blood aspirates via rAAV gene transfer as a means to enhance chondrogenic processes in the view of future implantation protocols to conveniently treat articular cartilage lesions. As hypertrophy and osteogenic differentiation were also induced by rAAV-hIGF-I here, regulation of transgene expression may need attention prior to translation in clinically relevant, orthotopic models of cartilage injury in vivo [42]. This might be achieved by employing tissue-specific (SOX9, type-II collagen) or regulatable (tetracycline-sensitive) promoters or by co-applying vectors coding for antihypertrophic factors [43] like FGF-2 [44], the family of SOX transcription factors [27, 45, 46], or an anti-Cbfa-1 siRNA [47]. However, no clear evidence of hypertrophy and terminal differentiation could be documented when providing the current rAAV IGF-I vector to experimental osteochondral lesions in the knee joints of rabbits [48]. Overall, the use of such manipulated blood samples may find value to allow for less invasive and less cumbersome treatments for the affected patients, avoiding the need for cell isolation, expansion and re-implantation in sites of cartilage injury.

Acknowledgements

This work 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. Tervelliger (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. We thank D. Zurakowski (Departments of Anesthesiology and Surgery, Boston Children’s Hospital, Harvard Medical School, Boston, MA, USA) for expert support in statistical analysis.

Conflict of interest

The authors confirm that there are no conflict of interests.

References

1. Frisch J, Orth P, Venkatesan JK, et al. Genetic modification of human peripheral blood aspirates using recombinant adenovirus vectors for articular cartilage repair with a focus on chondrogenic transforming growth factor-beta gene delivery. Stem Cells Transl Med. 2016; 6(1): 249–260. doi: 10.5966/sctm.2016-0149.

2. 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.

3. Madry H, Grun UW, Knutsen G. Cartilage repair and joint preservation: medical and surgical treatment options. Dtsch Arztebl Int. 2011; 108: 669–77.

4. Frisch J, Rey-Rico A, Venkatesan JK, et al. TGF-beta gene transfer and overexpression via rAAV vectors stimulates chondrogenic events in human bone marrow aspirates. J Cell Mol Med. 2016; 20: 430–40.

5. Slynarski K, Deszczyński J, Karpinski J. Fresh bone marrow and periosteum transplantation for cartilage defects of the knee. Transplant Proc. 2006; 38: 318–9.

6. Skowronski 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.

7. Enea D, Ceconi 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.

8. Gigante A, Ceconi S, Calcagno S, et al. Arthroscopic knee cartilage repair with covered microfracture and bone marrow concentrate. Artrosco Tech. 2012; 1: e175–80.

9. Skowronski J, Rutka M. Osteochondral lesions of the knee reconstructed with mesenchymal stem cells - results. Ortop Traumatol Rehabil. 2013; 15: 195–204.

10. Kim JD, Lee GW, Jung GH, et al. Clinical outcome of autologous bone marrow aspirates concentrate (BMAC) injection in degenerative arthritis of the knee. Eur J Orthop Surg Traumatol. 2014; 24: 1505–11.

11. Johnstone B, Yoo JU. Autologous mesenchymal progenitor cells in articular cartilage repair. Clin Orthop Relat Res. 1999; 367: S156–62.

12. WakiTani S, Yamamoto T. Response of the donor and recipient cells in mesenchymal cell transplantation to cartilage defect. Microsc Res Tech. 2002; 58: 14–8.

13. WakiTani S, 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: 595–609.

14. Brittleberg 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.

15. Egergelet C, Steinwachs MR, Reichelt A. The operative treatment of full thickness cartilage defects in the knee joint with
autologous chondrocyte transplantation. Saudi Med J. 2000; 21: 715–21.

16. Zvailler NJ, Marinova-Mutafchieva L, Adams G, et al. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res. 2000; 2: 477–86.

17. Raghunath J, Sutherland J, Salih V, et al. Chondrogenic potential of blood-acquired mesenchymal progenitor cells. J Plast Reconstr Aesthet Surg. 2010; 63: 841–7.

18. Chang PP, Selvaratnam L, Abbas AA, et al. Human peripheral blood derived mesenchymal stem cells demonstrate similar characteristics and chondrogenic differentiation potential to bone marrow derived mesenchymal stem cells. J Orthop Res. 2012; 30: 634–42.

19. Saw KY, Anz A, Merican S, et al. Articular cartilage regeneration with autologous peripheral blood progenitor cells and hyaluronic acid after arthroscopic subchondral drilling: a report of 5 cases with histology. Arthroscopy. 2011; 27: 493–506.

20. Saw KY, Anz A, Siew-Yoke Jee C, et al. Articular cartilage regeneration with autologous peripheral blood stem cells versus hyaluronic acid: a randomized controlled trial. Arthroscopy. 2013; 29: 684–94.

21. Skowronsji J, Skowronska R, Rutka M. Cartilage lesions of the knee treated with blood mesenchymal stem cells - results. Ortop Traumatol Rehabil. 2012; 14: 569–77.

22. 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.

23. Cucchiarini M, Henrichonet C, Mainard D, et al. New trends in articular cartilage repair. J Exp Orthop. 2015; 2: 8–15.

24. Cucchiarini M. Human gene therapy: novel approaches to improve the current gene delivery systems. Discov Med. 2016; 21: 495–506.

25. 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. Tissue Eng Part A. 2015; 21: 2460–71.

26. Frisch J, Venkatesan JK, Rey-Rico A, et al. Effects of rAAV-mediated FGF-2 gene transfer and overexpression upon the chondrogenic differentiation processes in human bone marrow aspirates. J Exp Orthop. 2016; 3: 16–25.

27. 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.

28. Frisch J, Venkatesan JK, Rey-Rico A, et al. Influence of insulin-like growth factor I overexpression via recombinant adenovector-mediated vector gene transfer upon the biological activities and differentiation potential of human bone marrow-derived mesenchymal stem cells. Stem Cell Res Ther. 2014; 5: 103–14.

29. Weimer A, Madry H, Venkatesan JK, et al. Benefits of recombinant adeno-associated virus (rAAV)-mediated insulin-like growth factor I (IGF-I) overexpression for the long-term reconstruction of human osteoarthritic cartilage by modulation of the IGF-I axis. Mol Med. 2012; 18: 346–58.

30. Griffin DJ, Orvedt KL, Nixon AJ, et al. Mechanical properties and structure-function relationships in articular cartilage repaired using IGF-I gene-enhanced chondrocytes. J Orthop Res. 2016; 34: 149–53.

31. Hemphill DD, Mcllwraith CW, Slayden RA, et al. Adeno-associated virus therapy vector scAAVIGF-I for transduction of equine articular chondrocytes and RNA-seq analysis. Osteoarthritis Cartilage. 2016; 24: 902–11.

32. Samulski RJ, Chang LS, Shenk T, et al. Helper-free stocks of recombinant adenovirus and adenovirus infected normal integration does not require viral gene expression. J Virol. 1989; 63: 3822–8.

33. 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.

34. 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.

35. Ahmed N, Vogel B, Rohde E, et al. CD45-positive cells of hematopoietic origin enhance chondrogenic marker gene expression in rat marrow stromal cells. Int J Mol Med. 2006; 18: 233–40.

36. Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther. 2007; 9: 204–13.

37. Fu WL, Zhou CY, Yu JK. A new source of mesenchymal stem cells for aricular cartilage repair: MSCs derived from mobilized peripheral blood share similar biological characteristics in vitro and chondrogenesis in vivo as MSCs from bone marrow in a rabbit model. Am J Sports Med. 2014; 42: 592–601.

38. 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.

39. Nonnenmacher M, Weber T. Intracellular transport of recombinant adeno-associated virus vectors. Gene Ther. 2012; 19: 649–58.

40. Osborn KD, Trippel SB, Mankin HJ. Growth factor stimulation of adult articular cartilage. J Orthop Res. 1989; 7: 35–42.

41. Koch H, Jadowiec JB, Campbell PG. Insulin-like growth factor-I induces early osteoblast gene expression in human mesenchymal stem cells. Stem Cells Dev. 2005; 14: 621–31.

42. Madry H, Ochi M, Cucchiarini M, et al. Large animal models in experimental knee sports surgery: focus on clinical translation. J Exp Orthop. 2015; 2: 9–20.

43. Frisch J, Cucchiarini M. Gene- and stem cell-based approaches to regulate hypertrophic differentiation in articular cartilage disorders. Stem Cells Dev. 2016; doi: 10.1089/scd.2016.0106.

44. 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.

45. 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.

46. 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.

47. Jeon SY, Park JS, Yang HN, et al. Co-delivery of SOX9 genes and anti-Cbfa-1 sRNA coated onto PLGA nanoparticles for chondrogenesis of human MSCs. Biomaterials. 2012; 33: 4413–23.

48. 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.