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Hydrogel-Guided, rAAV-Mediated IGF-I Overexpression Enables Long-Term Cartilage Repair and Protection against Perifocal Osteoarthritis in a Large-Animal Full-Thickness Chondral Defect Model at One Year In Vivo

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The regeneration of focal articular cartilage defects is complicated by the reduced quality of the repair tissue and the potential development of perifocal osteoarthritis (OA). Biomaterial-guided gene therapy may enhance cartilage repair by controlling the release of therapeutic sequences in a spatiotemporal manner. Here, the benefits of delivering a recombinant adeno-associated virus (rAAV) vector coding for the human insulin-like growth factor I (IGF-I) via an alginate hydrogel (IGF-I/AlgPH155) to enhance repair of full-thickness chondral defects following microfracture surgery after one year in minipigs versus control (*lacZ*/AlgPH155) treatment are reported. Sustained IGF-I overexpression is significantly achieved in the repair tissue of defects treated with IGF-I/AlgPH155 versus those receiving *lacZ*/AlgPH155 for one year and in the cartilage surrounding the defects. Administration of IGF-I/AlgPH155 significantly improves parameters of cartilage repair at one year relative to *lacZ*/AlgPH155 (semiquantitative total histological score, cell densities, matrix deposition) without deleterious or immune reactions. Remarkably, delivery of IGF-I/AlgPH155 also significantly reduces perifocal OA and inflammation after one year versus *lacZ*/AlgPH155 treatment. Biomaterial-guided rAAV gene transfer represents a valuable clinical approach to promote cartilage repair and to protect against OA.

1. Introduction

Articular cartilage, the strong gliding tissue covering the moving extremities of the bones, does not heal on its own if injured[1,2] and such cartilage defects may initiate the development of osteoarthritis (OA), a clinically and socioeconomically debilitating disease.[3] Among the different types of focal lesions, the smaller chondral defects are particularly important as they are highly prevalent in patients.[4] Marrow stimulation techniques such as microfracture are among the currently most preferred surgical procedures[5] to locally repopulate such lesions with chondrogenically active mesenchymal stromal cells (MSCs) originating from the subchondral bone.[6] However, the repair tissue resulting from their (osteo)chondral differentiation is of reduced mechanical quality and degenerates over time,[7] possibly expanding into previously not affected parts of the joint compartment that may ultimately lead to knee OA.[3,8]

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Material-based strategies as attractive tools to enhance cartilage repair have been explored to deliver therapeutic factors via controlled therapeutic protein release,[9,10] protein localization,[11] and protein binding,[12,13] with a particular focus on the highly chondroreparative insulin-like growth factor I (IGF-I).[14] Nevertheless, the efficacy of strategies based on the transfer of recombinant factors is generally hindered by their short pharmacological half-lives.[15] Biomaterial-guided delivery of chondrogenic gene sequences via gene transfer vectors to sites of cartilage injury is an attractive approach to enhance the healing of cartilage defects by controlling their release in an improved spatiotemporal manner while maintaining gene transfer efficacy in a natural microenvironment.[15] Such a gene therapy strategy may be more effective than the administration of biomaterial-free vectors prone to immune neutralization and/or to dissemination in nontarget locations and less invasive than indirect approaches based on the genetic manipulation of patients' cells prior to reimplantation.[15] While nanoparticles have been successfully employed to nonvirally deliver plasmidic IGF-I and microRNAs in models of cartilage lesions in vivo,[16] hydrogels may be also well adapted for translational applications in cartilage repair[17,18] as they may be derived from or composed of structural compounds similar to those present in the extracellular matrix (ECM) of the cartilage[17] and can be conveniently delivered using direct injections or minimally invasive arthroscopic interventions.[15,19,20] Interestingly, the use of hydrogels for the release of gene vectors in applications aiming at enhancing cartilage repair has been recently evoked[15,21,22] and systems based on clinically adapted alginate (AlgPH155) have been reported for their ability to target human MSCs via the controlled delivery of recombinant adeno-associated virus (rAAV) vectors (the most suited gene transfer vector for clinical applications compared with other viral or nonviral vectors)[15] coding for a reporter gene sequence in vitro.[23] The goal of the present study was to examine whether a therapeutic rAAV/AlgPH155 hydrogel system[23] may improve cartilage repair and reduce OA development in the long-term in a clinically relevant setting in a large animal. We selected human IGF-I due to its critical roles in MSC chondrogenesis,[24,25] stimulates articular chondrocyte proliferation, type-II collagen and proteoglycan synthesis,[26,27] and has been shown to be protective against OA development.[28,29] We tested the hypotheses that hydrogel-guided, rAAV-mediated IGF-I overexpression affords long-term cartilage repair and protects against inflammation and the potential development of perifocal OA in a minipig model of a full-thickness chondral defect treated with microfracture surgery after one year in vivo.

Our data show, for the first time to the best of our knowledge, that successful rAAV-mediated overexpression of IGF-I via AlgPH155-guided gene vector application (IGF-I/AlgPH155) significantly and safely enhances the repair of full-thickness chondral defects treated with microfracture in a large animal model after one year in vivo while also reducing the levels of perifocal OA and inflammation relative to control (reporter *lacZ*/AlgPH155) treatment. Such hydrogel-guided rAAV gene transfer may provide a potent, off-the-shelf therapeutic system for future translational strategies that aim at treating affected patients in a noninvasive manner.

2. Results

2.1. Effective Overexpression of IGF-I via rAAV/Alginate-Mediated Gene Transfer In Vitro and In Chondral Defects in Minipigs In Vivo

The candidate rAAV-hIGF-I/alginate hydrogel system (IGF-I/AlgPH155) was first tested at a best suited vector dose[23] to determine its ability to promote IGF-I expression in vitro in MSCs, the cells that repopulate focal cartilage defects,[30] as a proof of concept of the functionality of the approach relative to control conditions including an rAAV-*lacZ*/alginate hydrogel system (*lacZ*/AlgPH155), the hydrogel system without vector (AlgPH155), hydrogel-free vectors (IGF-I/- or *lacZ*/- treatments), and the absence of both hydrogel and rAAV (-/- treatment). Application of IGF-I/AlgPH155 led to significantly higher levels of IGF-I expression relative to all other conditions including administration of hydrogel-free rAAV-hIGF-I (IGF-I/- treatment), at any time point of the analysis (from day one to day 21, the longest time point evaluated) (up to 4.7-fold difference, always $P \leq 0.001$) (**Figure 1**). Of note, the IGF-I/- treatment as internal positive control led to higher levels of IGF-I expression relative to conditions where rAAV-hIGF-I was omitted (negative controls; up to twofold difference starting on day 5, always $P \leq 0.001$) while no significant difference was noted between the negative controls ($P \geq 0.160$) (Figure 1).

In light of such findings showing the benefits of delivering rAAV-hIGF-I via AlgPH155 over free vector application, the IGF-I/AlgPH155 hydrogel system was next directly applied to chondral defects in minipigs in a bilateral surgical approach[31] to examine its ability to promote IGF-I gene delivery and overexpression over time (one year) relative to the *lacZ*/AlgPH155 hydrogel system without additional test conditions to comply with the 3R principle.[32] An estimation of IGF-I expression in the repair tissue of the defects after one year via immunohistochemical analysis[27] showed significantly higher levels of IGF-I-positive cells in the defects treated with IGF-I/AlgPH155 relative to the *lacZ*/AlgPH155 defects (58.8% ± 13.9% vs 43.6% ± 14.4% IGF-I-immunostained cells, i.e., a 1.3-fold difference, $P = 0.034$) (**Figure 2**). Interestingly, IGF-I expression was also noted at this time point in the cartilage surrounding the IGF-I/AlgPH155 defects versus that of the *lacZ*/AlgPH155 defects (49.2% ± 3.3% vs 12.3% ± 2.5% IGF-I-immunostained cells, i.e., a 4-fold difference, $P \leq 0.001$) (insets of Figure 2). No IGF-I expression was observed at broader sites (synovium, quadriceps muscle adjacent to the patella, infrapatellar pad, subchondral bone marrow) (not shown).

2.2. Effects of IGF-I Overexpression via rAAV/Alginate-Mediated Gene Transfer on the Repair Processes in Minipig Chondral Defects over Time

The rAAV/alginate-treated defects were next processed to determine the effects of the IGF-I/AlgPH155 treatment on the macroscopic, histological, and biological repair processes in the defects over time relative to *lacZ*/AlgPH155.

After one year in vivo, there was no significant differences between the IGF-I/AlgPH155 and *lacZ*/AlgPH155 treatments either in the individual or total categories of a semiquantitative

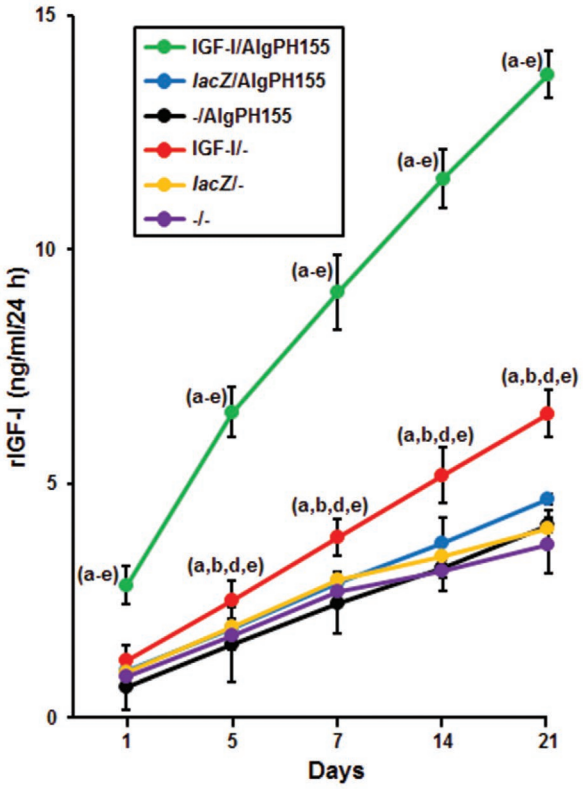

Figure 1. Detection of transgene (IGF-I) expression in MSCs treated with the rAAV/AlgPH155 hydrogel system. The IGF-I/AlgPH155 and *lacZ*/AlgPH155 hydrogel systems were directly applied to cultures of MSCs (25 µL for 2×10^4 cells, i.e., 3.6×10^5 transgene copies) for up to 21 days as described in the Experimental Section. Controls included the application of hydrogel without rAAV (AlgPH155), the administration of hydrogel-free rAAV vectors at similar vector doses (IGF-I/- or *lacZ*/- treatments), and the absence of both hydrogel and rAAV (-/- treatment). Cumulative IGF-I expression was measured at the denoted time points by ELISA as described in the Experimental Section. Statistically significant relative to *lacZ*/AlgPH155 (a), AlgPH155 (b), IGF-I/- (c), *lacZ*/- (d), and -/- (e) treatment.

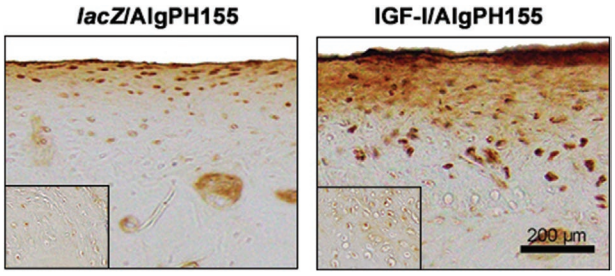

Figure 2. Detection of transgene (IGF-I) expression in the repair tissue of the rAAV/AlgPH155-treated minipig chondral defects. The IGF-I/AlgPH155 and *lacZ*/AlgPH155 hydrogel systems (25 µL per defect; 3.6×10^5 transgene copies) were implanted for one year in chondral defects (4 mm diameter, 5 mm depth) created in the lateral, distal femur trochlea of minipigs following standardized microfracture as described in the Experimental Section. The osteochondral units containing the defects were retrieved after one year and processed to monitor IGF-I expression by immunohistochemistry as described in the Experimental Section (insets: detection of IGF-I in the cartilage surrounding the defects; magnification ×10; all representative data).

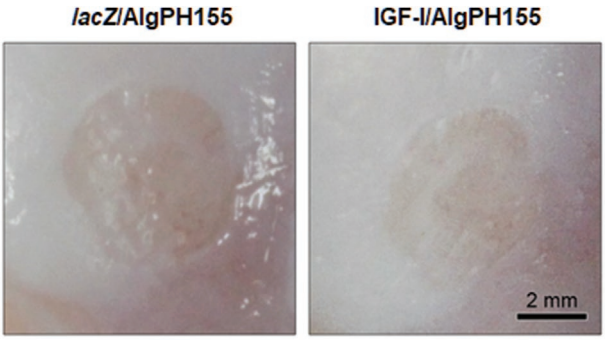

Figure 3. Macroscopic analysis of the repair tissue in the rAAV/AlgPH155-treated minipig chondral defects. The IGF-I/AlgPH155 and *lacZ*/AlgPH155 hydrogel systems were implanted in the minipig chondral defects as shown in Figure 2 and in the Experimental Section. The osteochondral units containing the defects were retrieved after one year for macroscopic evaluation as described in the Experimental Section (all representative data).

scoring system that grades the macroscopic repair of such defects[33] ($P \geq 0.145$) (**Figure 3** and **Table 1**), without signs of osteophyte formation, macroscopic synovitis, nor of immune cell infiltration (absence of CD3, CD11b, and HLA-DRα expression) at this time point in either treatment group examined (not shown).

Histological analysis performed on safranin O-stained sections using a validated inverse complex score that grades the microscopic structure of the repair tissue[34] revealed significant improvements in the individual parameters "filling of the defect," "cellular morphology," and "architecture of the defect surface" (all $P \leq 0.001$) at one year. Most importantly, the total histological score was significantly ($P = 0.015$) better in the IGF-I/AlgPH155 versus *lacZ*/AlgPH155 defects after one year, with a trend toward improved matrix staining and restrained architecture and subchondral bone plate ($P \leq 0.019$) (**Figure 4** and **Table 2**). An evaluation of the cell densities on safranin O/hematoxylin and eosin (H&E)-stained sections[25,27] at this time point identified significantly higher values in the repair tissue of the defects treated with IGF-I/AlgPH155 compared with those that received *lacZ*/AlgPH155 (1.2-fold difference, $P = 0.005$) (Figure 4 and **Table 3**). An estimation of the type-II collagen intensities on immunostained sections using a semi-quantitative score[35] revealed a significantly stronger deposition of this major ECM component in the repair tissue of defects that received IGF-I/AlgPH155 versus *lacZ*/AlgPH155 after

Table 1. Macroscopic analysis of the repair tissue in the rAAV/AlgPH155-treated minipig chondral defects.

Category	*lacZ*/AlgPH155	IGF-I/AlgPH155	*P* value
Color	1.38 ± 0.79	1.79 ± 0.81	0.145
Blood vessels	0.79 ± 0.60	1.13 ± 0.79	0.231
Surface	1.13 ± 0.66	1.08 ± 0.73	0.865
Filling	1.00 ± 0.94	1.38 ± 1.01	0.362
Adjacent cartilage	1.42 ± 0.89	1.79 ± 1.17	0.412
Total score	5.63 ± 2.62	7.08 ± 3.03	0.261

The macroscopic repair in the defects was evaluated using a semiquantitative score.[33] Values are given as mean ± standard deviation (SD).

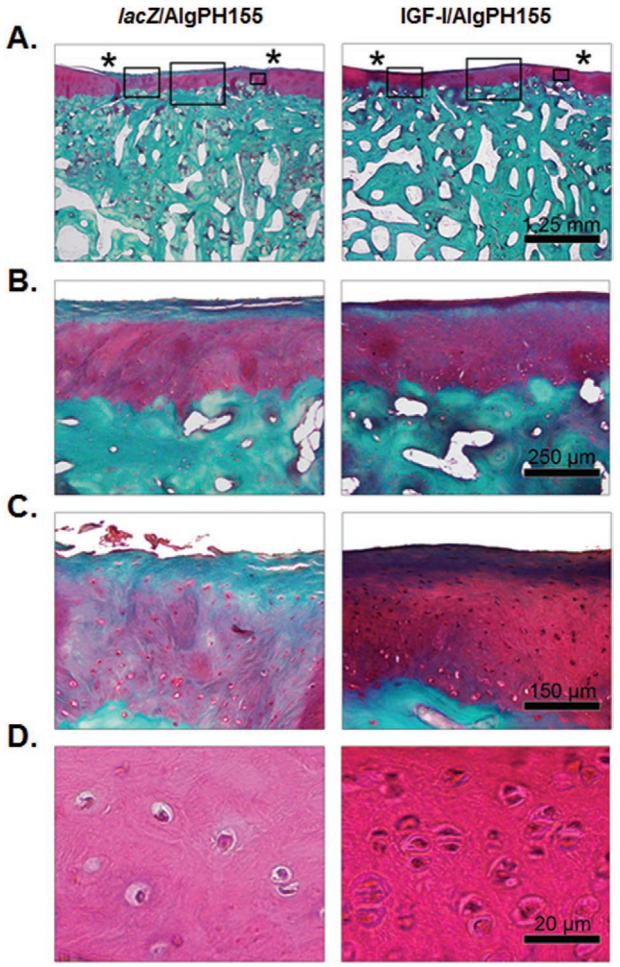

Figure 4. Histological analyses of the repair tissue in the rAAV/AlgPH155-treated minipig chondral defects. The IGF-I/AlgPH155 and *lacZ*/AlgPH155 hydrogel systems were implanted in the minipig chondral defects as shown in Figures 2 and 3 and in the Experimental Section. The osteochondral units containing the defects were retrieved after one year and processed for safranin O/H&E staining as described in the Experimental Section. The (*) indicate the integration sites and the boxes in (A) present the zones depicted in (B–D) (A: magnification ×2; B: magnification ×10; C: magnification ×20; D: magnification ×100; all representative data).

one year (1.3-fold difference, $P = 0.037$) (**Figure 5**A and Table 3). Instead, there was no difference in the deposition of type-I collagen[35] at this time point between the IGF-I/AlgPH155 and *lacZ*/AlgPH155 defects ($P = 0.203$) (Figure 5B and Table 3).

2.3. Effects of IGF-I Overexpression via rAAV/Alginate-Mediated Gene Transfer on the Inflammatory Responses and Perifocal OA in Minipig Chondral Defects over Time

The rAAV/alginate-treated defects were next examined to monitor a potential protection mediated by the IGF-I/AlgPH155 hydrogel system against inflammation and perifocal OA relative to *lacZ*/AlgPH155.

An analysis of OA features in the cartilage adjacent to the defects using an established inverse OA score[36] showed a significantly reduced overall perifocal OA around the defects treated with IGF-I/AlgPH155 relative to *lacZ*/AlgPH155 after one year (1.2-fold difference, $P = 0.048$), with trends toward improved individual parameters of adjacent cartilage structure, cell cloning, interterritorial staining, and tidemark/subchondral bone at this time point (up to 1.6-fold difference, $P \geq 0.081$) (**Figure 6** and **Table 4**).

Table 2. Histological analysis of the repair tissue in the rAAV/AlgPH155-treated minipig chondral defects.

Category	*lacZ*/AlgPH155	IGF-I/AlgPH155	*P* value
Filling of the defect	1.54 ± 0.89	0.92 ± 0.40	<0.001*
Integration	1.13 ± 0.33	1.19 ± 0.40	0.183
Matrix staining	2.56 ± 0.75	2.49 ± 0.84	0.561
Cellular morphology	1.85 ± 0.47	1.59 ± 0.49	<0.001*
Architecture of the defect	1.00 ± 0.65	1.31 ± 1.15	0.008*
Architecture of the surface	1.11 ± 0.36	0.86 ± 0.42	<0.001*
Subchondral bone plate	1.14 ± 1.13	1.42 ± 0.96	0.019*
Tidemark	4.00 ± 0.00	3.96 ± 0.20	0.072
Total score	14.32 ± 2.67	13.74 ± 2.56	0.015*

The repair processes in the defects were evaluated on safranin O-stained histological sections using a semiquantitative inverse complex cartilage repair score.[34] Values are given as mean ± standard deviation (SD). *Statistically significant versus *lacZ*/AlgPH155.

An evaluation of the inflammatory responses by immunohistochemistry revealed significantly decreased levels of IL-1β and TNF-α expression in the repair tissue of defects receiving IGF-I/AlgPH155 versus *lacZ*/AlgPH155 after one year (16% ± 3% vs 88% ± 4% IL-1β-immunostained cells, i.e., 5.6-fold decrease, and 4% ± 1% versus 86% ± 3% TNF-α-immunostained cells, i.e., a 22.8-fold decrease, always $P \leq 0.001$) (**Figure 7**). Similar results were noted when evaluating the levels of IL-1β and TNF-α expression in the cartilage surrounding the defects (7% ± 3% vs 67% ± 3% IL-1β-immunostained cells, i.e., 10.3-fold decrease, and 4% ± 2% vs 72% ± 3% TNF-α-immunostained cells, i.e., a 18-fold decrease, always $P \leq 0.001$) (insets of Figure 7).

3. Discussion

Noninvasive hydrogel-guided rAAV gene vector delivery is a potent emerging approach to promote cartilage repair via the

Table 3. Histomorphometric analysis in the repair tissue of the rAAV/AlgPH155-treated minipig chondral defects.

Category	*lacZ*/AlgPH155	IGF-I/AlgPH155	*P* value
Cell densities	296 ± 67	366 ± 36	0.005*
Type-II collagen	2.3 ± 0.5	3.0 ± 0.6	0.037*
Type-I collagen	1.6 ± 0.5	1.9 ± 0.6	0.203

The cell densities in the defects (cells mm^{-2}) were evaluated on safranin O-/H&E-stained histological sections. The deposition of type-II/-I collagen in the defects was evaluated on immunohistochemical sections using a semiquantitative score (0 = no immunoreactivity; 1 = significantly reduced immunoreactivity; 2 = moderately reduced immunoreactivity; 3 = similar immunoreactivity; 4 = stronger immunoreactivity compared with the controls).[35] Values are given as mean ± standard deviation (SD). *Statistically significant versus *lacZ*/AlgPH155.

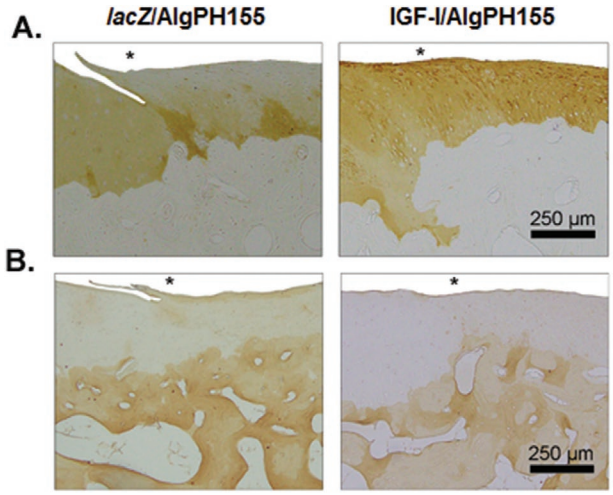

Figure 5. Immunohistochemical analyses of the expression of ECM components in the repair tissue of the rAAV/AlgPH155-treated minipig chondral defects. The IGF-I/AlgPH155 and *lacZ*/AlgPH155 hydrogel systems were implanted in the minipig chondral defects as shown in Figures 2–4 and in the Experimental Section. A,B) The osteochondral units containing the defects were retrieved after one year and processed to evaluate the deposition of type-II collagen (A) and type-I collagen (B) by immunohistochemistry as described in the Experimental Section (magnification ×4; all representative data).

spatiotemporal release of therapeutic sequences in sites of cartilage injury.[15,18] Such an approach might be more potent than strategies based on the delivery of short-lived recombinant factors[9–13] or of nonviral gene vectors[16] that are less stable and effective compared with rAAV and therefore much less adapted for clinical applications. In light of our recent findings showing the feasibility of providing a reporter (*lacZ*) rAAV vector via an alginate (AlgPH155) hydrogel to safely modify chondroreparative MSCs,[23] our goal was to examine the ability of such an rAAV/AlgPH155 hydrogel system to deliver a candidate gene coding for the highly chondrogenic and protective IGF-I[24–29] to durably improve the repair of clinically relevant chondral defects and prevent perifocal OA in a large-animal model (minipig) in vivo compared with control (reporter *lacZ*) alginate-guided gene application.

The results first indicate that local administration of the candidate IGF-I/AlgPH155 hydrogel system led to significantly higher levels of IGF-I production in MSCs in vitro relative to control *lacZ*/AlgPH155 hydrogel treatment over the whole period of evaluation (up to 21 days, the longest time point examined), confirming the potency and well maintenance of the rAAV-hIGF-I construct in the targets in vitro.[25,37] Most remarkably, the IGF-I/AlgPH155 hydrogel system was more potent to drive IGF-I overexpression via rAAV in MSCs than the rAAV-hIGF-I vector in its free form that supported lower levels of growth factor production similar to those reported by us in an earlier study.[25] Such superior amounts of IGF-I synthesis achieved via hydrogel-guided rAAV-hIGF-I gene transfer most likely result from an effective, sustained controlled release of rAAV from AlgPH155 as evidenced in our previous work using reporter rAAV/AlgPH155 constructs.[23] The current results also critically demonstrate that the IGF-I/AlgPH155 hydrogel system was capable of significantly inducing higher levels of

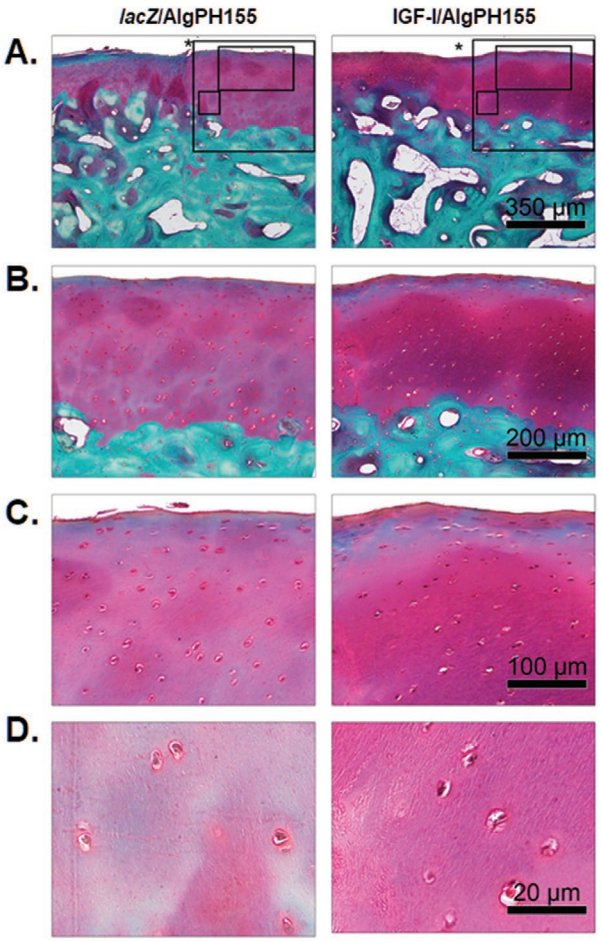

Figure 6. Histological analyses of the cartilage adjacent to the rAAV/AlgPH155-treated minipig chondral defects. The IGF-I/AlgPH155 and *lacZ*/AlgPH155 hydrogel systems were implanted in the minipig chondral defects as shown in Figures 2–5 and in the Experimental Section. The osteochondral units containing the defects were retrieved after one year and processed for safranin O staining as described in the Experimental Section. The (*) indicate the integration sites and the boxes in (A) present the zones depicted in (B–D) (A: magnification ×4; B: magnification ×10; C: magnification ×20; D: magnification ×40; all representative data).

IGF-I expression in minipig chondral defects in vivo compared with control *lacZ*/AlgPH155 treatment over an extended period of time (one year). These findings corroborate and expand

Table 4. Analysis of OA signs in the cartilage adjacent to the rAAV/AlgPH155-treated minipig chondral defects.

Category	*lacZ*/AlgPH155	IGF-I/AlgPH155	*P* value
Structure	1.18 ± 0.63	0.76 ± 0.68	0.081
Chondrocyte density	1.33 ± 0.47	1.39 ± 0.49	0.738
Cell cloning	3.54 ± 0.71	3.49 ± 0.81	0.946
Interterritorial staining	1.11 ± 0.32	1.07 ± 0.25	0.585
Tidemark, subchondral bone	1.83 ± 0.37	1.52 ± 0.69	0.159
Total score	9.00 ± 1.05	7.71 ± 2.46	0.048*

Signs of OA in the adjacent cartilage were evaluated on safranin O-stained sections using an established semiquantitative score.[36] Values are given as mean ± standard deviation (SD). *Statistically significant versus *lacZ*/AlgPH155.

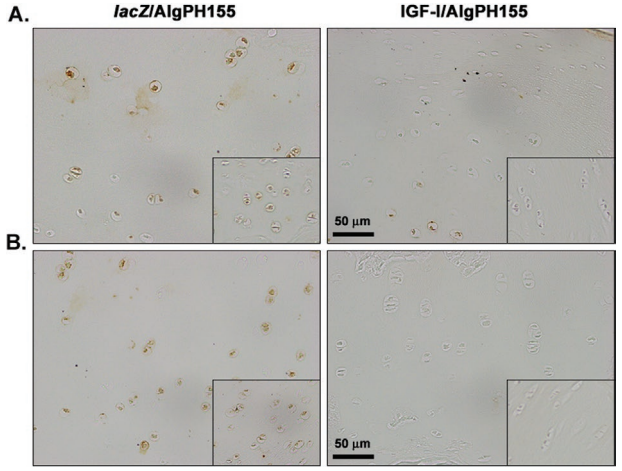

Figure 7. Immunohistochemical analyses of the inflammatory responses in the repair tissue and in the surrounding cartilage of the rAAV/AlgPH155-treated minipig chondral defects. The IGF-I/AlgPH155 and *lacZ*/AlgPH155 hydrogel systems were implanted in the minipig chondral defects as shown in Figures 2–6 and in the Experimental Section. A,B) The osteochondral units containing the defects were retrieved after one year and processed to evaluate the expression of IL-1β (A) and TNF-α (B) by immunohistochemistry in the repair tissue of the defects and in the surrounding cartilage (insets) as described in the Experimental Section (magnification ×40; all representative data).

our previous study showing the ability of rAAV-hIGF-I to support IGF-I overexpression in rabbit osteochondral defects for three weeks in vivo using a hydrogel-free gene vector delivery approach[27] and are probably resulting from the prolonged controlled release of rAAV from the AlgPH155 hydrogel as reported by us in vitro.[23] Such data also support the concept of employing rAAV for in vivo protocols over other classes of vectors that may be more immunogenic and functional only over short periods of time (some weeks) (adenoviral vectors)[38] and of using noninvasive, cell-free (rAAV) gene transfer approaches versus complex strategies based on the reimplantation of genetically modified cells.[39–43] Interestingly, expression of IGF-I in vivo here was also noted in the cartilage surrounding the IGF-I/AlgPH155 defects but not in the synovium, quadriceps muscle adjacent to the patella, infrapatellar pad, or subchondral bone marrow, possibly due to the effective release of rAAV from the hydrogel in the defects and at close distance, but not to an undesirable vector spreading and dissemination at broader sites.

The data next demonstrate that after one year, there was no evidence of deleterious effects of either the IGF-I/AlgPH155 or *lacZ*/AlgPH155 treatment in minipig chondral defects in vivo, without signs of osteophytes, synovitis, or of immune cell infiltration. This observation confirms the safety of the rAAV/hydrogel system and of rAAV-mediated IGF-I gene transfer as previously noted when testing the current (reporter) rAAV/hydrogel system in vitro[23] and when applying the rAAV-hIGF-I vector to rabbit osteochondral defects in a hydrogel-free form.[27]

The results further reveal that local application of IGF-I/AlgPH155 significantly and persistently enhanced the processes of cartilage repair in minipig chondral defects for at least one year in vivo relative to control *lacZ*/AlgPH155 treatment, with significantly improved microstructural repair, significantly enhanced cellularity, and significantly higher ECM deposition (stronger type-II collagen deposition, trend toward more proteoglycans, absence of undesirable type-I collagen), in good agreement with the properties of the growth factor.[26,44] These findings expand our previous observations in rabbit osteochondral defects using the rAAV-hIGF-I vector in a hydrogel-free form for three weeks,[27] even though type-I collagen expression was reduced with IGF-I in the rabbits[27] probably due to the difference in time points evaluated (three weeks vs one year here), as the amounts of vector added were in the same range in both studies (2×10^5 vs 3.6×10^5 transgene copies here). For comparison, significant benefits of IGF-I overexpression were reported in complex cell-based approaches for only 28 weeks in rabbit osteochondral defects using IGF-I-transfected chondrocytes using a poly(glycolic acid) (PGA) scaffold[41] and for eight months in horse chondral defects treated with rAAV-transduced chondrocytes.[42,43] Our current off-the-shelf, cell-free strategy is also more adapted and durable than the use of recombinant IGF-I as tested by other groups who applied the factor at a high very high dose (25 μg vs up to ≈13.5 ng here, i.e., ≈1850-fold difference) to achieve therapeutic effects in rabbit osteochondral defects for only 12 weeks using an oligo(poly(ethylene glycol) fumarate)/gelatin composite hydrogel[45] and in horse chondral defects for 6–8 months using fibrin alone[26] or chondrocyte–fibrin composites.[46] The reduced parameters of "defect architecture" and "subchondral bone plate" were caused by a large osteochondral cleft in one of the treated defects that was not excluded from scoring.

Finally, the present findings reveal that the IGF-I/AlgPH155 hydrogel system was capable of significantly reducing the long-term development of perifocal OA in the cartilage adjacent to the minipig chondral defects, with trends toward improved microscopic structure after one year and decreased expression of detrimental proinflammatory mediators (IL-1β, TNF-α) in the defects and surrounding cartilage relative to the *lacZ*/AlgPH155 condition, possibly due to the overexpression of IGF-I from the candidate hydrogel system at a close distance, in good agreement with the protective effects of the growth factor against OA pathomechanisms (cartilage degradation, inflammation)[28,29,47,48] and with our previous work showing a reduction of OA changes in the cartilage surrounding rabbit osteochondral defects treated with IGF-I-transfected chondrocytes/PGA constructs for 28 weeks.[41]

4. Conclusion

In summary, and for the first time to the best of our knowledge, the present study demonstrates the potential benefits of hydrogel (alginate)-guided therapeutic rAAV gene delivery to improve cartilage repair in vivo while advantageously reducing perifocal OA and inflammation over an extended period of time versus control treatment. A limitation of the study is the absence of a quantitative biochemical analysis and of the assessment of the mechanical function of the repaired cartilage and adjacent tissues. As complete regeneration was not achieved in the conditions tested here, coadministration of other candidates might be desirable, like the chondroreparative and anti-hypertrophic cartilage-specific sex-determining region-type high mobility

group box 9 (SOX9) transcription factor.[49–52] This might be performed by simultaneous or independent gene transfer of various rAAV via alginate since cotransduction of such vectors can be successfully established without viral interference.[53,54] Taken together, the current findings highlight the value of the rAAV/AlgPH155 hydrogel system as a future direct, minimally-invasive, off-the-shelf solution for enhanced surgical therapies to repair cartilage defects in clinical scenarios. From a future clinical perspective, such a hydrogel-guided, rAAV-mediated overexpression of therapeutic gene sequences will ideally be performed during arthroscopic marrow stimulation given the symmetric pattern of enhanced long-term cartilage repair and protection against perifocal OA.

5. Experimental Section

Reagents: All reagents were from Sigma (Munich, Germany) unless otherwise indicated. The sodium alginate (GRINDSTED AlgPH155, molecular weight = 140 kDa, mannuronic to glucuronic (M:G) ratio = 1:5, viscosity = 350–550 mPas s) was purchased at Danisco (Copenhagen, Denmark). The AAVanced Concentration Reagent was obtained at System Bioscience (Heidelberg, Germany). The anti-IGF-I (NBP2-16929) and anti-IL-1β (3553) antibodies were from Novus Biologicals (Centennial, CO, USA), the anti-type-II collagen (II4C11) and anti-CD11b (Ly-40) antibodies from Acris (Hiddenhausen, Germany), the anti-type-I collagen (COL-1) antibody from Abcam (Cambridge, UK), the anti-CD3 (PC3/188A) antibody from Santa Cruz Biotechnology (Heidelberg, Germany), the anti-HLA-DRα (TAL-1B5) antibody from Dako (Agilent, Waldbronn, Germany), and the anti-TNF-α (abx069528) antibody from Abbexa (Cambridge, UK). The biotinylated secondary antibodies and the ABC reagent were from Vector Laboratories (Burlingame, CO, USA). The IGF-I Quantikine ELISA was from R&D Systems (Wiesbaden, Germany).

Cell Culture: Bone marrow aspirates were obtained from the distal femurs of patients undergoing total knee arthroplasty (n = 6). All patients provided informed consent prior to inclusion in the study, which was approved by the Ethics Committee of the Saarland Physicians Council. All protocols were in compliance with the Helsinki Declaration. Bone-marrow-derived mesenchymal stromal cells (MSCs) were isolated via standard protocols.[23,25] Briefly, aspirates (≈15 mL per patient) were washed, centrifugated in Dulbecco's modified Eagle medium (DMEM), and the pellet was resuspended in red blood cell lysing buffer and DMEM (1:1). The mixture was washed, pelleted, and resuspended in DMEM, 10% fetal bovine serum, 100 U mL^{-1} penicillin, and 100 µL mL^{-1} streptomycin (growth medium) for plating in T75 flasks at 37 °C, 5% CO_2. The medium was changed after 24 h and replaced by growth medium, 1 ng mL^{-1} recombinant FGF-2, with medium change every 2–3 days. Cells were replated at an 85% density and used at passage 1–2.

Plasmids and rAAV Vectors: The constructs were derived from pSSV9, an AAV-2 genomic clone.[25,27,55] rAAV-*lacZ* carries the *Escherichia coli* β-galactosidase (*lacZ*) gene and rAAV-hIGF-I a human IGF-I cDNA, both under the control of the cytomegalovirus immediate-early (CMV-IE) promoter.[25,27] The vectors were packaged as conventional (not self-complementary) vectors using a helper-free, two-plasmid transfection system using the packaging plasmid pXX2 and the Adenovirus helper plasmid pXX6 in the 293 packaging cell line.[27] The vector preparations were purified with the AAVanced Concentration Reagent according to the manufacturer's instructions and titered by real-time PCR,[23,25,27] averaging 10^{10} transgene copies mL^{-1} (viral particles to functional vectors = 500/1).

Preparation of Hydrogels Containing the rAAV Vectors: The systems were prepared using 3% alginate (AlgPH155) in PBS.[23] The rAAV-loaded capsules were prepared by directly mixing the alginate solution (100 µL) with the rAAV vector dispersions (270 µL; 5.4×10^6 transgene copies) with subsequent crosslinking in calcium chloride (102×10^{-3} M) at room temperature in a 2 mL^{-1} tube using a syringe with a stainless steel 21 gauge needle[23] to generate the rAAV/alginate hydrogel systems (IGF-I/AlgPH155 and *lacZ*/AlgPH155).

Evaluations In Vitro: Human MSCs were placed in 24-well plates (2×10^4 cells per well) and directly incubated for up to 21 days with the rAAV/alginate hydrogel systems (IGF-I/AlgPH155, *lacZ*/AlgPH155) in growth medium. Controls included the application of AlgPH155 hydrogel system without rAAV, the administration of hydrogel-free rAAV vectors (rAAV-hIGF-I and rAAV-*lacZ*) at a best suited vector dose[23] similar to those applied next in vivo (25 µL, i.e., 3.6×10^5 transgene copies, MOI = 20), and the absence of alginate hydrogel system and vector treatment. IGF-I expression was measured by ELISA as previously described).[25] Briefly, the cultures were washed twice and placed for 24 h in serum-free medium. Supernatants were collected and centrifugated at the denoted time points to remove debris and processed by ELISA according to the manufacturer's recommendations with measurements performed on a GENios spectrophotometer/fluorometer (Tecan, Crailsheim, Germany).[25]

Full-Thickness Trochlea Chondral Defect Model: The animal experiments were performed in accordance with the German legislation on the protection of animals approved by the Saarland University Animals Committee (27/2017) and the EU Directive 2010/63/EU for animal experiments. The minimal sample size of 8 was calculated as previously described.[31,56] Healthy, skeletally mature female Aachener minipigs (age 18–22 months, average body weight (BW): 42.75 ± 6.54 kg; Gerd Heinrichs, Heinsberg, Germany) were fed by standard diet and received water ad libitum. After 12 h fasting, sedation was induced with an intramuscular injection of 30 mg ketamin per animal (Ketanest S, Pfizer, Berlin, Germany), 2 mg xylazine per animal (Rompun, Bayer, Leverkusen, Germany), and 1 mg atropine per animal (B. Braun, Melsungen, Germany). After endotracheal intubation to receive 20 mL 2% propofol (AstraZeneca, Wedel, Germany), the balanced general anesthesia was maintained by inhalation of 1.5% isoflurane (Baxter, Unterschleißheim, Germany) and intravenous propofol (6–20 mg kg^{-1} BW/h). Using a bilateral medial parapatellar approach, a miniarthrotomy in 60 °C flexion was performed without a need for patellar luxation. With a dermatological punch (Kai Europe, Solingen, Germany), standardized circular full-thickness chondral defects of 4 mm in diameter were outlined in the weight-bearing area of distal lateral femoral trochlea of each joint. After meticulously removing the articular cartilage within the defect with a curette including all calcified cartilage down to the subchondral bone plate that was left intact, three uniform microfracture holes were introduced (1.2 mm diameter, 5 mm depth) using a custom-made microfracture awl with a straight trihedral cutting tip and a penetration stop.[57] The hydrogel systems (IGF-I/AlgPH155 or *lacZ*/AlgPH155) (25 µL per defect; 3.6×10^5 transgene copies)[23] were implanted in the defects that were prior blotted dry using a spatula where they adhered to the subchondral bone plate treated with microfracture. Following implantation, the joint was taken through several ranges of motion to test the stability of the hydrogel. A detachment was never noted. Then, the arthrotomy was closed in layers with interruptured sutures, skin closure was achieved with surgical staples, and a spray bandage was applied. Postoperative pain management consisted of a fentanyl pain patch with a release rate of 100 µg h^{-1} for 72 h and—if needed—4 mg kg^{-1} BW carprofen (Rimadyl, Pfizer). The animals were sacrificed in general anesthesia one year postoperatively. Pictures of the entire defect area were obtained under standardized illumination conditions for the macroscopic evaluation. The samples were next placed in 4% formalin for 24 h and stored in 70% ethanol prior to decalcification in 5% formic acid, trimming, and embedding in paraffin for histological and immunohistochemical evaluations including scoring assessments.

Macroscopic Analysis: Postoperative pictures of the trochlear defects were independently evaluated by three blinded observers for macroscopic grading of the articular defect repair using an inverse macroscopic scoring system (20 = no repair; 0 = normal articular cartilage).[33]

Histological Analysis: Histological sections (thickness: 3 µm) were stained with safranin O/fast green (safranin O) to detect proteoglycans and with H&E for cellularity.[34,58] A total of 96 slides (12 per defect) were analyzed by two blinded observers using an inverse complex cartilage

repair score (0 = normal articular cartilage; 31 = no repair tissue).[34] The cell densities (cells mm^{-2}) in the defects were assessed on safranin O-/H&E-stained sections by two blinded observers using four defined image excerpts at magnification ×20.[25,27] Samples were examined under light microscopy (Olympus BX45, Hamburg, Germany).

Immunohistochemical Analysis: For the evaluation of the immunoreactivity to IGF-I, type-II/-I collagen, CD3 (T lymphocytes), CD11b (activated macrophages), HLA-DRα (class II major histocompatibility complex antigens), IL-1β, and TNF-α, histological sections were deparaffinized and placed in 0.3% hydrogen peroxide for 30 min.[25,27] The sections were then washed with PBS and incubated in 0.1% trypsin for 10 min at 37 °C, washed with PBS, and placed in blocking buffer (3% bovine serum albumin in PBS) for 30 min at room temperature.[27] The antibodies were then diluted in blocking buffer and applied to the sections for 12 h at 4 °C (anti-IGF-I: 1/20; anti-type-II/-I collagen: 1/90; anti-CD3: 1/2000; CD11b: 1/500; HLA-DRα: 1/500; IL-1β: 1/100; TNF-α: 1/45).[27] Control conditions lacking primary antibodies were also evaluated to check for secondary immunoglobulins. The sections were next washed with PBS and incubated with biotinylated secondary antibodies (1/200) for 1 h at room temperature.[27] The sections were washed with PBS and incubated with avidin–biotin-peroxidase reagent for 30 min at room temperature, washed with PBS, and revealed with diaminobenzidine.[27] Samples were examined under light microscopy (Olympus BX45). Expression of IGF-I, CD3, CD11b, HLA-DRα, IL-1β, and TNF-α was assessed by two blinded observers by measuring the percentage of immunostained cells for each marker per total cell numbers using three standardized random sites in the defects with the SIS analySIS program (Olympus) and Adobe Photoshop (Adobe Systems, Unterschleissheim, Germany,[27] with analyses also performed for some markers (IGF-I, IL-1β, TNF-α) in the surrounding cartilage and at broader sites (synovium, quadriceps muscle adjacent to the patella, infrapatellar pad, subchondral bone marrow). Expression of type-II and -I collagen was graded by two blinded observers using a semiquantitative score (0 = no immunoreactivity; 1 = significantly reduced immunoreactivity; 2 = moderately reduced immunoreactivity; 3 = similar immunoreactivity; 4 = stronger immunoreactivity compared with the controls)[37] at three standardized random sites with the SIS analySIS program (Olympus) and Adobe Photoshop (Adobe Systems). For type-II collagen, the adjacent cartilage was defined as the normal control and for type-I collagen, the subchondral bone plate was used as the control.

Evaluation of Perifocal OA: To assess perifocal OA in the articular cartilage adjacent to the chondral defects, safranin O-stained sections ($n = 6$ per animal) were scored by two blinded observers using an inverse OA score (0 = no OA; 25 = severe OA).[36] Samples were examined under light microscopy (Olympus BX45).

Statistical Analysis: Each condition in vitro was carried out in triplicate in three independent experiments with all samples and with all defects in vivo. Data are presented as mean ± standard deviation, mean absolute difference between the study groups (mean difference), and 95% confidence interval of the mean absolute difference. First, a Shapiro-Wilk test was performed to evidence normal distribution of the data. Generalized estimating equations (GEE) within an analysis of variance (ANOVA) framework were used to account for multiple values per defect. Having only one value per defect, the Student's *t*-test was applied. Calculations were performed using Stata (StataCorp LCC, College Station, TX, USA), SPSS version 23.0 (SPSS Inc. Chicago, IL, USA), and GraphPad Prism version 8.0.0 (GraphPad Software, Inc. San Diego, CA, USA). *P* values < 0.05 were considered statistically significant.

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Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

H.M. and M.C. designed the study. J.M., H.M., A.R.-R., J.K.V., G.S., S.S.-M., X.C., W.M., D.Z., M.D.M., M.W.L., and M.C. performed the experiments. A.R.-R. and J.K.V. performed the material development. J.M., H.M., A.R.-R., J.K.V., L.G., G.S., S.S.-M., X.C., W.M., D.Z., M.D.M., M.W.L., and M.C. contributed to the data analysis and interpretation. J.M., H.M., and M.C. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Research data are not shared.

Keywords

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