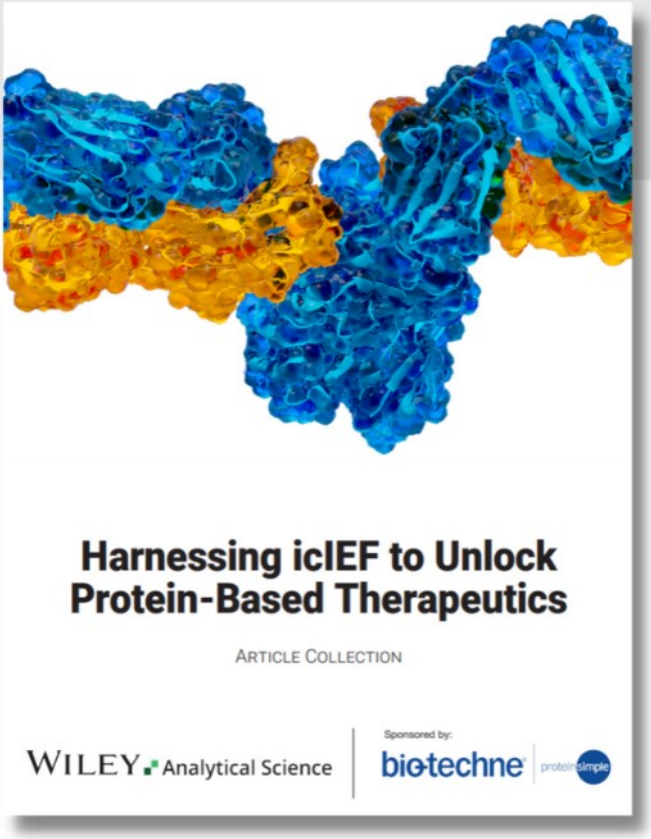
Harnessing icIEF to Unlock Protein-Based Therapeutics



Read the new Article Collection

Keep up to date with the latest developments in biotherapeutics and the range of treatments for various diseases with our latest article collection. Find out how imaged cIEF (icIEF) technique is essential for quality control and analytical development of these drugs, as it accurately determines the surface charge of lipid nanoparticles and the charge heterogeneity of proteins and antibodies.

This article collection aims to provide you with more information on these techniques and technologies, helping you further your research in this field.

bio-techne® | protein simple

WILEY

ADVANCED THERAPEUTICS
www.advtherap.com

Phage Display Identified Peptide with Selectivity for Human Osteoarthritic Chondrocytes

*Ivone M. Martins, Raphaël F. Canadas, Hélder Pereira, Joana Azeredo, Rui L. Reis, Joaquim M. Oliveira, and Helena S. Azevedo**

Osteoarthritis (OA) is one of the most common joint disorders in western populations, affecting millions of people worldwide and with a rising incidence as life expectancy continues to increase. Current therapies for OA management fail to halt the progressive degradation of articular cartilage, urging the need for more effective therapies to improve cartilage function and enhance patient's quality of life. Through phage display technology, biopanning on a population of heterogenous chondrocyte cells isolated from six different OA donors, and using a random 12-amino acid peptide phage library, a peptide selective for human OA chondrocytes (GFQMISNNVYMR) is identified. A twofold increase in fluorescence intensity is observed for OA chondrocytes, compared to normal chondrocytes, when cells are incubated with the identified peptide conjugated to a fluorescent label, being selectively internalized by OA cells. The identified peptide can be further modified and exploited for developing early diagnostic of OA and/or improve drug delivery to target cells through peptide-drug conjugates.

1. Introduction

The development of targeted therapies is one of the major goals of current research in biomedicine to improve treatment outcomes for diseases affecting a large portion of the population.

Osteoarthritis (OA) is a complex degenerative and debilitating joint disease characterized by a progressive degradation of articular cartilage, osteophyte formation, and stiffening of the joints, leading to loss of mobility and function accompanied by chronic pain.[1] It affects more than 500 million people worldwide, being the leading cause of disability in older adults and also frequent due to sport lesions,[2] posing a huge economic burden.[3]

Nowadays, disease management involves pain medication with nonsteroidal

I. M. Martins, R. F. Canadas, H. Pereira, R. L. Reis, J. M. Oliveira, H. S. Azevedo
3B's Research Group
I3Bs—Research Institute on Biomaterials
Biodegradables and Biomimetics
University of Minho
Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine
AvePark
Parque de Ciência e Tecnologia
Zona Industrial da Gandra
Barco, Guimarães 4805-017, Portugal
E-mail: hazevedo@i3s.up.pt

I. M. Martins, R. F. Canadas, H. Pereira, R. L. Reis, J. M. Oliveira, H. S. Azevedo
ICVS/3B's—PT Government Associate Laboratory
Guimarães, Braga 4710-057, Portugal

I. M. Martins, J. Azeredo
CEB—Centre of Biological Engineering
University of Minho
Braga 4710-057, Portugal

I. M. Martins, J. Azeredo
LABBELS—Associate Laboratory
Guimarães, Braga Portugal

R. F. Canadas
Tech4MED™
R. 25 de Abril, Ronfe, Guimarães, Braga 4805-369, Portugal

R. F. Canadas
FMUP–Faculdade de Medicina
Universidade do Porto
Alameda Prof. Hernâni Monteiro
Porto 4200-319, Portugal

H. Pereira
Orthopedic Department
Centro Hospitalar Póvoa de Varzim-Vila do Conde
Largo da Misericórdia, Póvoa de Varzim 4490-421, Portugal

J. Azeredo, H. S. Azevedo
i3S—Instituto de Investigação e Inovação em Saúde
Universidade do Porto
Porto 4200-135, Portugal

J. Azeredo, H. S. Azevedo
INEB—Instituto de Engenharia Biomédica
Universidade do Porto
Rua Alfredo Allen, 208, Porto 4200-135, Portugal

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adtp.202300263

DOI: 10.1002/adtp.202300263

anti-inflammatory drugs (NSAIDs) or analgesics to relieve the symptoms, with joint replacement surgery the final stage of treatment.[4] However, the guidelines from the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) encourage the development of effective disease-modifying osteoarthritis drugs (DMOADs).[5] DMOADs are drugs that modify the underlying OA pathophysiology to prevent or reduce long-term disability and offer potential symptomatic relief.[6] Moreover, given its heterogeneity and the moderate effect of symptomatic treatments, there is an unmet clinical need to develop targeted therapies for OA.[7]

Articular cartilage is a unique connective avascular and non-innervated tissue that shows little or no potential for intrinsic repair following injury, where chondrocytes are the only residing cells. Chondrocytes are responsible for the synthesis and degradation of the extracellular matrix (ECM), a dynamic 3D network of macromolecules that provides structural support for the cells and tissues.[8] In OA, chondrocytes can undergo hypertrophic differentiation, converting ECM components, from type II collagen and aggrecan-rich to type I and type X collagen-rich matrix, and increasing the expression of multiple matrix metalloproteinases.[9]

Chondrocytes and cartilage ECM have been exploited as targets for localized therapy.[10] Using phage display technology, several peptides were identified that bind specifically to collagen II α1 (WYRGRL),[11] using denuded bovine cartilage, rabbit (DWRVIIPPRPSA, RLDPTSYLRTFW)[12] and mice (HDSQLEALIKFM)[13] chondrocytes.

In these studies, the identified peptides were discovered using nonhuman normal cartilage and chondrocytes, while peptides with affinity toward human OA chondrocytes have not been reported to date.

Here, we describe the use of phage display technology to select a peptide targeting human OA cartilage cells.

2. Results and Discussion

2.1. Characterization of hACs Isolated from Normal and OA Cartilage Specimens

The in vitro phage display selection experiment was conducted on human articular chondrocytes (hACs) isolated from surgical specimens of a cohort of six OA patients and two non-OA donors (Table S1, Supporting Information). To confirm the disease phenotype of the articular cartilage donors, histological characterization was performed. Compared to normal cartilage, OA cartilage tissue exhibited structural alterations, with an irregular surface, broken by fissures and fibrillation, decreased cellularity (reduced in situ chondrocyte volume), and an increasing number of empty and enlarged *lacunae* (Figure S1, Supporting Information), characteristic features of the disease.[14]

Isolated hACs were cultured on 2D monolayers and their morphology and proliferation analyzed (**Figure 1**A). A lower cell number is observed for OA chondrocytes compared to normal cells, indicating a lower proliferative rate. Both cells showed an elongated fibroblast-like phenotype with OA cells being smaller in size (Figure 1B).

In general, OA cartilage cells showed a decreased expression of collagen type I, aggrecan, and sox9, while only Donor 1 had twofold increase of collagen type I and Donor 2 had half-fold increase of sox9 (Figure 1C). Half of the samples analysed (from Donor 2, 5, and 6) also showed decreased expression level of collagen type X. For collagen type II, the expression levels from OA cartilage samples varied greatly, where a 17-fold increase was observed for Donor 7 and ≈0.5-fold decrease for Donors 1 and 5 (Figure 1C). These changes in marker gene expression suggest that the cells from Donor 1, 2, 3, and 7 were undergoing hypertrophic differentiation, but probably being at different stages.[9] The presence and expression of certain surface markers, including CD10, CD26, CD44, and CD95, were also assessed. CD10 (neprilysin) and CD26 (dipeptidyl peptidase IV) were both reported to be expressed in human articular chondrocytes and their expression decreases with the worsening of OA.[15] CD26 is involved in interactions with the ECM proteins, collagen, and fibronectin,[16] while CD44, a transmembrane glycoprotein and principal cell surface receptor for hyaluronan (HA), participates in the uptake and degradation of this important component of articular cartilage ECM[17] and has been described to be associated with progressive knee OA joint damage in articular cartilage.[18] CD95 (Fas) is a cell membrane surface receptor that, when bound to its ligand (FasL) or agonistic anti-Fas antibody to the Fas receptor, activates chondrocyte apoptosis.[19,20] Moreover, Fas expression was reported to be increased in OA and aged cartilage, compared to normal cartilage.[21,22]

OA hACs showed slightly decreased expression levels of CD44, compared to normal hACs, and ninefold and 0.25-fold decrease of CD10 and CD26, respectively (Figure 1D and Figure S2, Supporting Information). The expression levels of CD95 on OA cells were slightly higher than normal. The expression alterations of CD95, CD10, and CD26 are similar to the ones reported in other studies, while CD44 expression was reported to be decreased in OA chondrocytes from patients with greater levels of OA severity.[15,18,21]

2.2. Phage Display Selection of Chondrocyte-Binding Peptides on Moderate-to-Severe OA Phenotypes

One counter-selection and three rounds of biopanning were applied as a general screening workflow of BRASIL—biopanning and rapid analysis of selective interactive ligands, on all OA primary cells (**Figure 2**A) and using the M13 phage library displaying random linear peptides composed of 12 natural amino acids and fused to the phage coat pIII protein at the C-terminus (Figure 2C). Among the six samples of OA cells analyzed, an enrichment of potential binding peptides was only obtained for Donor 7 (Figure 2B). Twelve randomly selected phage clones were sequenced and all displayed the same sequence fused to the N1 and N2 ectodomains of pIII: N-GFQMISNNVYMR-C. To verify the incidence of this sequence in previous studies, the linear peptide was searched against the database from the Biopanning Data Bank.[24] No similar sequence was found against 33 097 peptides grouped into 3562 sets (search undertaken on July 2023). When extending the search to the database of target-unrelated peptides for phage display studies (TUPs) using SAROTUP (Scanner and Reporter of Target-Unrelated Peptides), no homologous sequence was found.[25]

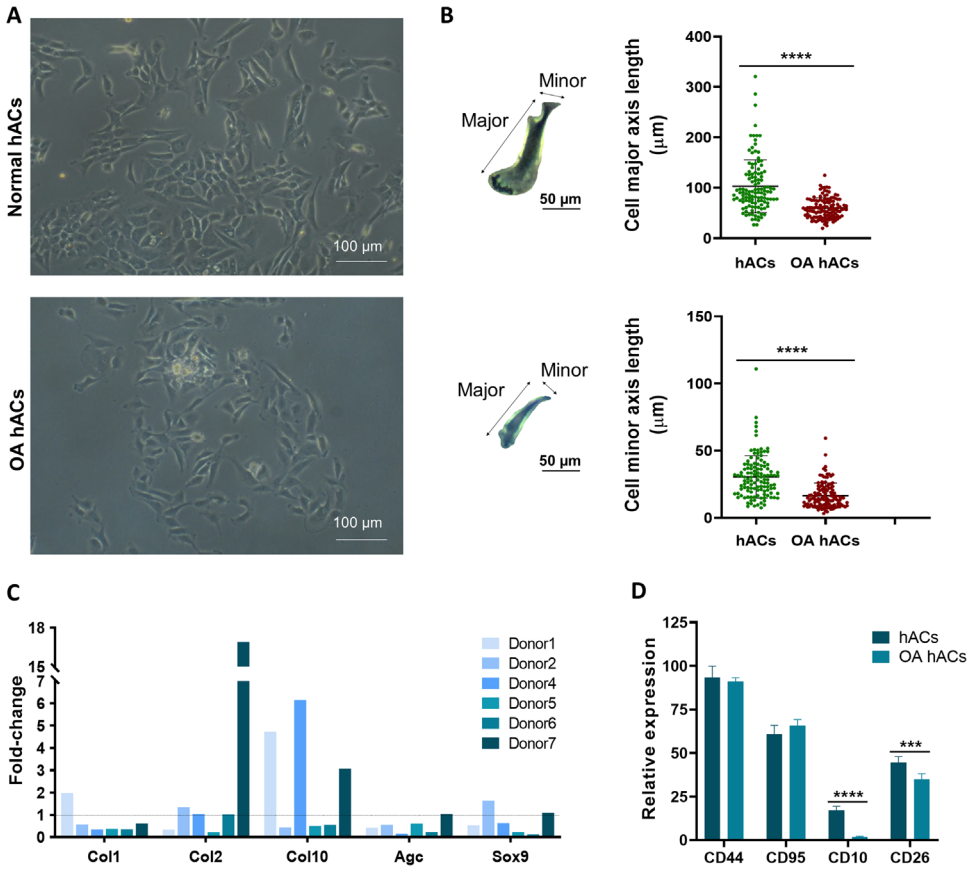


Figure 1. Characterization of hACs isolated from normal and OA cartilage specimens. A) Representative optical microscopy images of hACs cultured as 2D cell monolayers after isolation at first passage. B) Measurement of cell length: 240 cells were analyzed, including normal ($n = 120$) and OA chondrocytes ($n = 120$), for major and minor cell size measurement using ImageJ. Significant differences are shown as: ****$p < 0.0001$. C) Gene-level differential on OA chondrocytes versus normal (control) chondrocytes. Data are shown as fold change of cells from OA cartilage samples ($n = 6$) as compared to gene expression of cells from normal cartilage samples ($n = 2$). Each gene was processed in triplicate. RNase-free water was used as a negative control. In each sample, the transcript expressions of target genes were normalized to the average expression of the endogenous housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) value and the gene expression in healthy chondrocytes. The quantification of fold changes in gene expression was performed using the $2^{-\Delta\Delta Ct}$ methods.[23] D) Surface marker expression of CD44, CD95, CD10, and CD26 on normal and OA chondrocytes, presented as percentage of expression (percentage values are the average of all cytometry assays for normal and OA samples, from the gated events of each condition donor/marker). The results in percentage are shown as the mean of three independent assays for each of the OA samples compared to the normal ones. Significant differences are shown as: ***$p \leq 0.001$ and ****$p < 0.0001$.

To exclude any peptides reported to bind chondrocytes, a search by chondrocytes as target (Target ID1318 and ID990) was also performed and only three entries were found, corresponding to primary mouse (BiopanningDataSet ID2167)[13] and rabbit (BiopanningDataSet ID1548 and ID1549)[12] chondrocytes. The similarity of the obtained peptide with other sequences identified by other groups for cartilage-related targets, either human or animal chondrocytes, or cartilage tissue components, such as collagen type II, was further checked. Three peptides (WYRGRL, DPHFHL, and RVMLVR BiopanningDataSet ID486) specific for collagen type II were reported by Hubbell's group on denuded bovine cartilage grafts.[11] No similarity was observed among these sequences. Likewise, the peptide identified by Pi et al.[12] using cartilage pieces from rabbit as target (DWRVIIPPRPSA BiopanningDataSet ID1549), was not homologous to the peptide found in this study, suggesting a sequence specific for human OA hACs.

The found consensus peptide sequence (OAhAC-Pep, GFQMISNNVYMR), as well as a scrambled sequence containing the same amino acid composition but in different order (Scr-Pep, YQMMSVNIFGRN), was chemically synthesized with a FAM (fluorescein-5(6)-carbonyl) fluorescent label at the lysine (K) side chain at the C-terminus: H-GFQMISNNVYME-GGGS-KKK(FAM)-amide and H-YQMMSVNIFGRN-GGGS-KKK(FAM)-amide, to enable their detection in cell cultures (Table S3 and Figure S3, Supporting Information). The peptide modifications were made at the C-terminus to leave the N-terminus free for interaction, since the peptides are fused to the phage pIII coat protein via the C-terminus (Figure 2C,D).

2.3. Selectivity of Identified Peptide for OA Chondrocytes

Though the identity of the potential cell-surface binding partner to the 12-mer peptide (OAhAC-Pep) selected from the

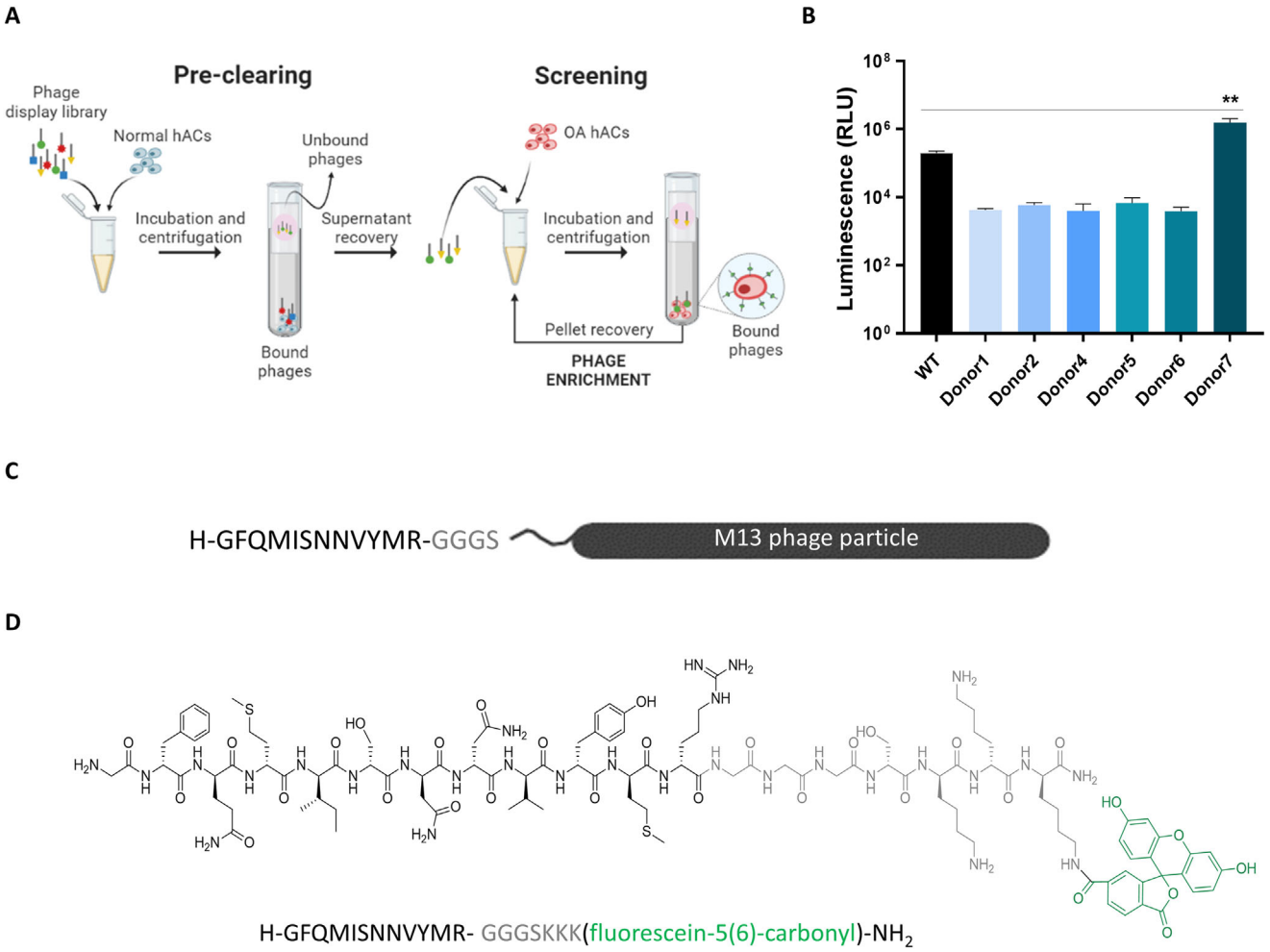


Figure 2. Identification of peptides selective for OA chondrocytes by phage display biopanning and their subsequent molecular design. A) Schematic illustrating the BRASIL methodology: pre-incubation of normal human articular chondrocytes (hACs) with the phage display library (preclearing step) followed by incubation of the cleared library with OA target cells (screening step). Additional rounds of biopanning were performed for phage enrichment. B) Detection of positive phage pools by enzyme-linked immunosorbent assay (ELISA). Phage-peptides bound to the target cells were detected by an anti-M13 antibody coupled with horseradish peroxidase (HRP) that degrades the HRP substrate *o*-phenylenediamine dihydrochloride (OPD), providing a signal measured by relative light units (RLUs). Phage pools were analyzed in triplicate. A negative control (assay performed with the same conditions but without target cells) was used to subtract the background noise. M13KE without any displayed peptide was used as a positive control, establishing a lower limit on the quantitative analysis performed via RLUs measurement. Significant differences are shown as: $****p \leq 0.0001$. Identified peptide sequence (GFQMISNNVYMR): C) as displayed on the phage surface, fused to the pIII protein via the C-terminus and connected via the GGGS linker. D) As redesigned in this study, keeping similar configuration as displayed on the phage (with free N-terminus) and bearing a fluorophore on the side chain of a terminal lysine (K) for fluorescence detection. Image A was created with BioRender.com.

biopanning experiments remains unknown, its binding selectivity to OA hACs over normal hACs was next examined (**Figure 3** and Figures S4–S6, Supporting Information). A higher fluorescence signal (green) was observed in the cytoplasm of OA cells when incubated with OAhACs-Pep, suggesting the peptide internalization by the cells (Figure 3A). Nonetheless, these studies were conducted with cells cultured in monolayer which may have affected the way proteins are exposed at the cell surface, promoting the peptide internalization. The biopanning experiments were conducted using cells in suspension (Figure 2A), probing the whole cell area.

The current treatment options for OA are limited, with no effective DMOADs, but gene therapy has been considered safe and effective for OA treatment. Although no gene products have been approved for OA,[26] recent studies are revealing new targets for therapeutic interventions, such as small interfering RNA (siRNA) therapy for suppressing transcription factors regulating apoptosis (e.g., nuclear factor kappa B, NF-κB),[27] which requires infiltration into the dense and avascular cartilage matrix and intracellular delivery. In fact, the inefficient delivery of drugs into deeper cartilage layers, and to resident chondrocytes, has been identified as major bottleneck in OA treatment. Therefore, the use of cartilage-penetrating carriers (e.g., cationic nanocarriers or functionalized with amphipathic cationic cell-penetrating peptides) has been shown to improve the delivery and efficacy of OA therapies.[28] The identified sequence (GFQMISNNVYMR) has

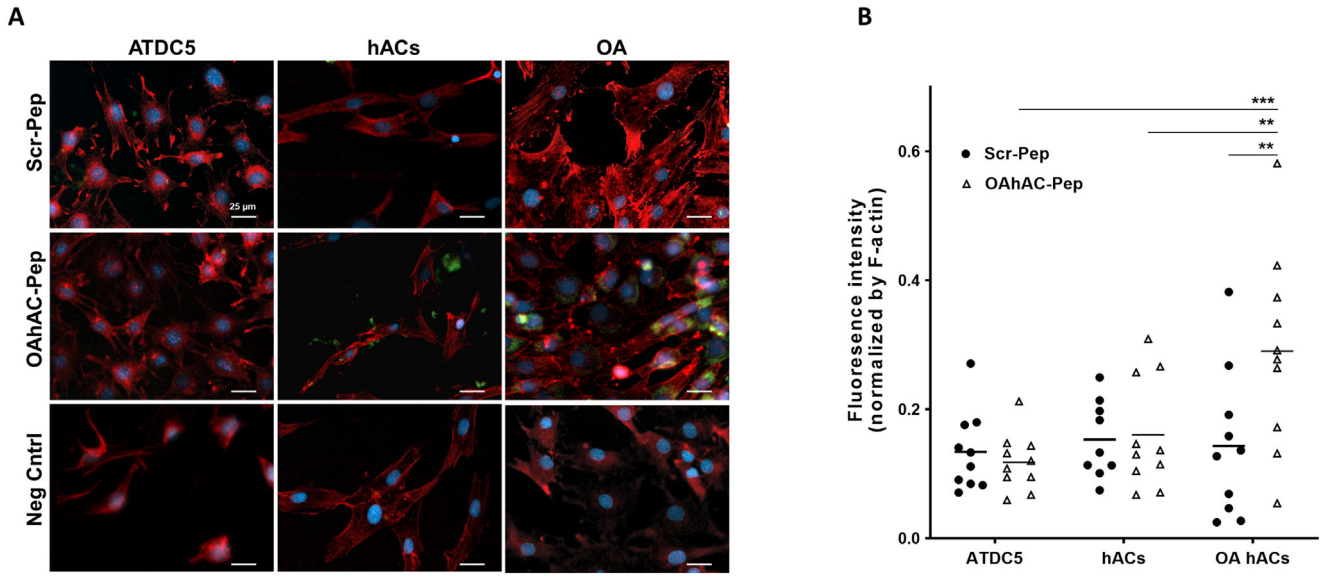


Figure 3. Validation of peptide selectivity for OA chondrocytes. A) Selectivity of OAhAC-Pep examined by fluorescence microscopy. Cartilage-derived cells (OA hACs and normal hACs) were incubated with Scr-Pep and OAhAC-Pep at concentration of 5×10^{-6} M. The negative control is cells without peptide. A green fluorescent signal is observed only when OA cells are incubated with OAhAC-Pep. B) The fluorescence intensity of the peptide was normalized by F-actin (10 replicates) in all images acquired. Significant differences are shown as: $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

positive net charge (+2) at neutral pH, but it may not be sufficient to penetrate anionic cartilage. Nonetheless, the original sequence can be further modified at C-terminus to incorporate cationic residues, as already done in this study for the cell-binding studies (Figure 2D). For therapeutic agents acting directly on chondrocytes (e.g., catabolic or anabolic factors), targeted delivery is required.[10] The internalization of the peptide observed in the cell-binding studies suggests its ability in facilitating the intracellular delivery of therapeutic agents (e.g., siRNA) capable of suppressing catabolic genes (e.g., proteolytic enzymes) to inhibit matrix degradation and preventing further progress of the disease.

3. Conclusion

A novel peptide sequence, GFQMISNNVYMR, selective to human OA articular chondrocytes was identified by phage display. After being validated in dose-dependent cytotoxicity assays, as well as in functional analysis to elucidate the binding mechanism and potential biological effect, this sequence can be further tested (e.g., diffusion studies into human cartilage specimens) and optimized to enhance the delivery of drugs to target OA chondrocytes and modulate their anabolic or catabolic activities.

4. Experimental Section

Primary Cell Isolation and Characterization: Human articular cartilage specimens from eight different donors were collected at Centro Hospitalar da Póvoa de Varzim e Vila do Conde (Portugal) under a pre-established agreement with the Hospital´s Ethical Committee (CE.CHPVVC 1427–06052011). All samples were processed for cell isolation within 12 h after collection (Table S1, Supporting Information). OA articular cartilage tissue was obtained from patients who underwent knee arthroplasty and presenting Kellgren–Lawrence radiological grading of OA ≥ III.[29] Controls of normal articular cartilage tissue were obtained in the consequence of acute traumatic injuries in patients without OA and all tissue specimens were obtained after patient's informed consent.

Histological analysis was performed to characterize and confirm the progression of the disease. For that, specimens were fixed, dehydrated, embedded in paraffin and sliced into 5 µm sections. Tissue slices were then stained with hematoxylin and eosin (H&E) for later observation. The stained sections were observed under a reflected/transmitted light microscope (Axioimager Z1M, Zeiss), and images were taken with a digital camera (Axion MRc5, Zeiss).

Human articular chondrocyte cells (hACs) were isolated with an enzymatic cocktail composed of 0.25% trypsin (Alfagene), 400 U mL^{-1} collagenase I (Biochrom), 1000 U mL^{-1} collagenase II (Biochrom), and 1 mg mL^{-1} hyaluronidase type II (Sigma) for 1 h at 37 °C under gentle stirring.[30] After digestion, hAC cells were cultured as 2D monolayer in 6-well plates in complete culture medium [Coon's F-12 modified with 2.5 g L^{-1} $NaHCO_3$ without L-glutamine medium (Biotecnomica), supplemented with 10% fetal bovine serum (FBS, Fisher Scientific), 1% v/v antibiotic/antimycotic solution (ATB, Alfagene) and 1% L-glutamine (Alfagene)] in a 37 °C humidified atmosphere with 5% CO_2. Cells were observed by microscopy using a Zeiss Primovert (Zeiss, Germany) inverted microscope and their length was measured using FIJI (12) (NIH, v. 1.52e, http://imagej.nih.gov/ij). Briefly, a total of 240 cells comprising normal (n = 120) and OA chondrocytes (n = 120) were assessed for major and minor cell size measurement.

RNA Isolation and Gene Expression Analysis of Collagen I, II, and X, Aggrecan, and Sox9: Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed to assess the expression profile of some commonly used markers of chondrocyte cells, namely, collagen I, II, and X, aggrecan, and sox9.[31,32] Total RNA was extracted from hACs at passage 1 and reverse transcribed to cDNA. RT-qPCR was carried out to detect amplification variations using PerfeCta SYBR Green FastMix (Quanta Biosciences), using a Mastercycler ep realplex gradient S equipment (Eppendorf). The analysis of the results was performed with realplex software (Eppendorf Mastercycler, Applied Biosystems). The primer sequences were obtained from RTPrimerDB and PrimerBank databases, and synthesized by MWG Biotech (Table S2, Supporting Information).

Characterization of Chondrocyte Cells by Flow Cytometry: Flow cytometry was used to assess the expression of chondrocyte surface markers, including CD10, CD26, CD44, and CD95.[15,18,21] Normal and OA

chondrocytes at 90% confluence growth were detached and resuspended in sterile phosphate-buffered saline (PBS) 1X. Approximately 2×10^5 cells were incubated with the respective fluorescent conjugated monoclonal antibody: CD10-APC, CD26-FITC, CD95-FITC (eBioscience), and CD44-PE (BD Pharmingen), at the concentration of 0.25, 1, 0.5, and 1 µg per test, respectively, for 20 min at room temperature in the dark. From each sample, 20 000 events were analyzed by FACS scan (BD FACSCalibur, BD Biosciences) and data were assessed by CELLQuest V3.3 software (BD Biosciences).

Selection of Cell-Binding Peptides Targeting OA hACs using Phage Display: A random 12-mer (with a theoretical diversity of 1×10^{13} variants) M13 phage library (New England Biolabs) was used for screening against chondrocytes using the Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL) methodology, adapted from previously reported studies.[33,34] Briefly, in a preclearing step, hACs were first incubated with the phage library and the unbound phages collected. For the biopanning screening, the unbound fraction was incubated with OA hACs from individual donors. The cell-phage pairs were separated by centrifugation and resuspended in 100 µL of 1 M Tris-HCl buffer (pH 9.1) and stored at 4 °C. An aliquot of the eluted phage was subsequently reapplied to a new batch of OA cells (same passage), in a total of three rounds without amplification.

Identification of Cell-Binding Peptides: ELISA was performed using an adapted protocol from the NEB Ph.D. Phage Display Libraries Instruction Manual.[35,36] One row of ELISA plate wells for each phage pool from the third biopanning round of each of the six OA samples was coated with target cells in complete culture medium, followed by incubation at 37 °C with 5% humidity overnight, to allow cell attachment. The culture medium was removed and the plate washed 3 times with Tris buffered saline with Tween 20 1X (TBST). Cells were incubated with the phage pool at a concentration of 1×10^8 PFU/well under agitation for 2 h. The plate was washed three times with TBS 1X with Tween 20 (TBST) prior to incubation with horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody (GE Healthcare), diluted in 1% of bovine serum albumin (BSA) in TBS 1X to the final dilution of 1:5000, and incubated at room temperature for 1 h. Subsequently, the plate was washed with TBST three times and the freshly prepared o-phenylenediamine dihydrochloride (OPD, Thermo Scientific) substrate for HRP detection was added to each well and incubated for 15 min. A parallel ELISA assay, with no target (negative control), was also performed to distinguish real target-binders from plastic binders (background noise). The wild-type M13KE phage was used as baseline control. The plates were read in a single tube luminometer (GloMax 20/20, Promega). Individual clones from the phage pool of donor 7 were sent for sequencing (Macrogen), following the procedure described in NEB Ph.D. Phage Display Libraries Instruction Manual and in other reports.[35–37]

Search for Chondrocyte Binding Peptides: The identified peptide sequence and the keyword "chondrocyte" (Target ID 1318 and 990) were used to search against the Biopanning Data Bank. SAROTUP was used to search the sequence against target-unrelated peptide bank.

Analysis of Peptide Binding and Selectivity using Fluorescence Microscopy: The consensus peptide sequence found from the biopanning experiments (OA hAC-Pep, GFQMISNNVYMR), as well as a scrambled sequence containing the same amino acid composition but in different order (Scr-Pep, YQMMSVNIFGRN, generated by the tool available at http://www.mimotopes.com/peptideLibraryScreening.asp?id=97), were chemically synthesized by Bachem (Switzerland) (Figure 2D and Table S3, Supporting Information). The peptides were synthetized with a FAM (fluorescein-5(6)-carbonyl) fluorescent label at the lysine (K) side chain at the C-terminus: H-GFQMISNNVYME-GGGS-KKK(FAM)-amide and HYQMMSVNIFGRN-GGGS-KKK(FAM)-amide, to enable their detection in cell cultures. The peptide modifications were made at the C-terminus to leave the N-terminus free for interaction, since the peptides were fused to the phage pIII coat protein via the C-terminus (Figure 2C,D). The GGGS linker was integrated in the sequence because Ph.D.−12 phage clones of the phage display library contained the GGGS linker sequence between the displayed peptide and the coat protein. Three additional lysine residues were included in the sequence to improve the solubility of the peptides and facilitate the conjugation of the fluorescent label. The designed peptide displayed the binding sequence (GFQMISNNVYME) unmodified and separated from the anchor side, similar to the mode it was displayed on the phage surface. hACs from OA (donor #7) and normal (donor #13) cartilage and the ATDC5 chondrogenic cell line derived from Mouse 129 teratocarcinoma AT805 (used as a negative control), were seeded onto plastic coverslips (SPL Life Sciences), and allowed to grow in complete culture medium in a 37 °C humidified atmosphere with 5% CO_2. After 24 h, cells were fixed with paraformaldehyde, to maintain their structure, and blocked with bovine serum albumin. Cells were then incubated individually with each FAM-peptides, diluted in PBS to 5×10^{-6} M (this concentration was chosen after protocol optimization using serial dilutions from 5×10^{-6} to 50×10^{-6} M), for 1 h at 4 °C.[35] Cells were then washed with ice-cold PBS and incubated with DAPI (4,6-diamidino-2-phenylindole dihydrochloride, 1:5000 dilution, Biotium) and Phalloidin–Tetramethylrhodamine B isothiocyanate (1:500 dilution, Sigma) at 37 °C for 15 min to stain the cell nuclei and cytoskeleton (F-actin), respectively. The images were acquired by a Reflected Light Axio Imager Z1m Zeiss Microscope at 20X magnification and analyzed using ImageJ. All images correction for background and intensity threshold were set as the same, and peptide quantification was normalized by the area occupied by phalloidin (F-actin) inside the cells.

Statistical Analyses: Data obtained from RT-qPCR analysis and flow cytometry were presented as a mean ± standard deviations (SD) of three independent experiments. Statistical analysis was performed using the nonparametric Kruskal–Wallis test and unpaired *t* test with Welch's correction. Values of $p < 0.05$ were considered to determine statistically significant differences between the groups. The gene expression fold change of collagen I, II, and X, aggrecan, and transcriptional factor SOX-9 was normalized to the average expression of the endogenous housekeeping gene GAPDH value and the gene expression in normal chondrocyte cells using $2^{-\Delta\Delta Ct}$ methods.[23]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the project PTDC/EBB-BIO/114523/2009 and the strategic funding of UIDB/04469/2020 unit, by the European Regional Development Fund (ERDF) through the Operational Competitiveness Programme "COMPETE" (FCOMP-01-0124-FEDER-014758). The authors thank FCT the strategic funding of UID/BIO/04469/2013 unit and RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462). R.F.C. and J.M.O. are also thankful to FCT, Fundo Europeu de Desenvolvimento Regional (FEDER), and Programa Operacional Competitividade e Internacionalização (POCI) for funding the B-Liver Project (PTDC/EMD-EMD/29139/2017). R.F.C. and J.M.O. thank the financial support provided under the project "HEALTH-UNORTE: Setting-up biobanks and regenerative medicine strategies to boost research in cardiovascular, musculoskeletal, neurological, oncological, immunological, and infectious diseases," reference NORTE-01-0145-FEDER-000039, funded by the Norte Portugal Regional Coordination and Development Commission (CCDR-N), under the NORTE2020 Program. H.S.A. thanks the MOBILIsE project funded by the European Union's Horizon 2020 research and innovation programme under Grant Agreement no. 951723. The authors thank the staff of Centro Hospitalar Póvoa de Varzim-Vila do Conde for their cooperation and Dr Franklin L. Nobrega from the University of Southampton for the advice and valuable discussions during the course of the work.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

H.S.A. conceived the study; H.S.A. and I.M.M. designed the experiments; I.M.M. and R.F.C. performed the experiments; I.M.M., R.F.C., J.M.O., and H.S.A. analyzed the data; H.P. collected and provided the cartilage specimens; I.M.M. and H.S.A. wrote the manuscript; All authors reviewed the manuscript.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords

diagnostic, human chondrocytes, osteoarthritis, peptides, phage display, targeted therapy

Received: July 21, 2023
Revised: October 4, 2023
Published online:

[1] B. Abramoff, F. E. Caldera, *Med. Clin. North Am.* **2020**, *104*, 293.
[2] F. Migliorini, N. Maffulli, A. Pintore, J. Ernst, J. Eschweiler, F. Hildebrand, M. Betsch, *Sports Med. Arthrosc. Rev.* **2022**, *30*, 118.
[3] V. P. Leifer, J. N. Katz, E. Losina, *Osteoarthritis Cartilage* **2022**, *30*, 10.
[4] C. VanAtta, L. A. Perry, A. Crase, A. Swierz, S. Wenzinger, *U.S. Pharm.* **2023**, *48*, 17.
[5] W. M. Oo, D. J. Hunter, *Clin. Exp. Rheumatol.* **2019**, *37*, 135.
[6] Y. Cho, S. Jeong, H. Kim, D. Kang, J. Lee, S. B. Kang, J. H. Kim, *Exp. Mol. Med.* **2021**, *53*, 1689.
[7] X. Cai, S. Yuan, Y. Zeng, C. Wang, N. Yu, C. Ding, *Front. Pharmacol.* **2021**, *12*, 645842.
[8] H. Akkiraju, A. Nohe, *J. Dev. Biol.* **2015**, *3*, 177.
[9] P. Singh, K. B. Marcu, M. B. Goldring, M. Otero, *Ann. N. Y. Acad. Sci.* **2019**, *1442*, 17.
[10] I. M. Martins, R. L. Reis, H. S. Azevedo, *ACS Chem. Biol.* **2016**, *11*, 2962.
[11] D. A. Rothenfluh, H. Bermudez, C. P. O'Neil, J. A. Hubbell, *Nat. Mater.* **2008**, *7*, 248.
[12] Y. X. Z. Pi, J. Shi, J. Zhu, W. Chen, C. Zhang, W. Gao, C. Zhou, Y. Ao, *Biomaterials* **2011**, *32*, 6324.
[13] C. S. Cheung, J. C. Lui, J. Baron, *J. Orthop. Res.* **2013**, *31*, 1053.
[14] W. Waldstein, G. Perino, S. L. Gilbert, S. A. Maher, R. Windhager, F. Boettner, *J. Orthop. Res.* **2016**, *34*, 135.
[15] G. Lapadula, F. Iannone, C. Zuccaro, M. Covelli, V. Patella, G. Lobianco, V. Pipitone, *Clin. Exp. Rheumatol.* **1995**, *13*, 143.
[16] I. De Meester, S. Korom, J. Van Damme, S. Scharpe, *Immunol. Today* **1999**, *20*, 367.
[17] A. Aruffo, I. Stamenkovic, M. Melnick, C. B. Underhill, B. Seed, *Cell* **1990**, *61*, 1303.
[18] F. J. Zhang, W. Luo, S. G. Gao, D. Z. Su, Y. S. Li, C. Zeng, G. H. Lei, *Mod. Rheumatol.* **2013**, *23*, 1186.
[19] H. A. Kim, F. J. Blanco, *Curr. Drug Targets* **2007**, *8*, 333.
[20] K. Kuhn, D. D. D'Lima, S. Hashimoto, M. Lotz, *Osteoarthritis Cartilage* **2004**, *12*, 1.
[21] A. T. Pennock, C. M. Robertson, B. C. Emmerson, F. L. Harwood, D. Amiel, *Arthritis Rheum.* **2007**, *56*, 1529.
[22] R. Todd Allen, C. M. Robertson, F. L. Harwood, T. Sasho, S. K. Williams, A. C. Pomerleau, D. Amiel, *Osteoarthritis Cartilage* **2004**, *12*, 917.
[23] K. J. Livak, T. D. Schmittgen, *Methods* **2001**, *25*, 402.
[24] B. He, G. Chai, Y. Duan, Z. Yan, L. Qiu, H. Zhang, Z. Liu, Q. He, K. Han, B. Ru, F. B. Guo, H. Ding, H. Lin, X. Wang, N. Rao, P. Zhou, J. Huang, *Nucleic Acids Res.* **2016**, *44*, D1127.
[25] B. He, H. Chen, N. Li, J. Huang, *Int. J. Biol. Sci.* **2019**, *15*, 1452.
[26] W. Zhang, H. Ouyang, C. R. Dass, J. Xu, *Bone Res.* **2016**, *4*, 15040.
[27] H. Yan, X. Duan, H. Pan, N. Holguin, M. F. Rai, A. Akk, L. E. Springer, S. A. Wickline, L. J. Sandell, C. T. Pham, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E6199.
[28] B. C. Geiger, S. Wang, R. F. Padera Jr., A. J. Grodzinsky, P. T. Hammond, *Sci. Transl. Med.* **2018**, *10*, 469.
[29] J. H. Kellgren, J. S. Lawrence, *Ann. Rheum. Dis.* **1957**, *16*, 494.
[30] R. C. Pereira, C. Gentili, R. Cancedda, H. S. Azevedo, R. L. Reis, *Methods Mol. Biol.* **2011**, *695*, 167.
[31] R. Dreier, *Arthritis Res. Ther.* **2010**, *12*, 216.
[32] G. Lapadula, F. Iannone, *Semin. Arthritis Rheum.* **2005**, *34*, 9.
[33] R. Derda, S. Musah, B. P. Orner, J. R. Klim, L. Li, L. L. Kiessling, *J. Am. Chem. Soc.* **2010**, *132*, 1289.
[34] R. J. Giordano, M. Cardo-Vila, J. Lahdenranta, R. Pasqualini, W. Arap, *Nat. Med.* **2001**, *7*, 1249.
[35] D. Ferreira, A. P. Silva, F. L. Nobrega, I. M. Martins, C. Barbosa-Matos, S. Granja, S. F. Martins, F. Baltazar, L. R. Rodrigues, *Sci. Rep.* **2019**, *9*, 3958.
[36] F. L. Nobrega, D. Ferreira, I. M. Martins, M. Suarez-Diez, J. Azeredo, L. D. Kluskens, L. R. Rodrigues, *BMC Cancer* **2016**, *16*, 881.
[37] V. L. Silva, D. Ferreira, F. L. Nobrega, I. M. Martins, L. D. Kluskens, L. R. Rodrigues, *PLoS One* **2016**, *11*, e0161290.