



HAL
open science

Direct Perfusion Improves Redifferentiation of Human Chondrocytes in Fibrin Hydrogel with the Deposition of Cartilage Pericellular Matrix

Alexandre Dufour, Frédéric Mallein-Gerin, Emeline Perrier-Groult

► **To cite this version:**

Alexandre Dufour, Frédéric Mallein-Gerin, Emeline Perrier-Groult. Direct Perfusion Improves Redifferentiation of Human Chondrocytes in Fibrin Hydrogel with the Deposition of Cartilage Pericellular Matrix. Applied Sciences, In press, 10.3390/app11198923 . hal-03357277

HAL Id: hal-03357277

<https://hal.science/hal-03357277>

Submitted on 28 Sep 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

Article

Direct Perfusion Improves Redifferentiation of Human Chondrocytes in Fibrin Hydrogel with the Deposition of Cartilage Pericellular Matrix

Alexandre Dufour, Frédéric Mallein-Gerin and Emeline Perrier-Groult *

Laboratory of Tissue Biology and Therapeutic Engineering (LBTI), Institute for Biology and Chemistry of Proteins, CNRS, UMR 5305, 69000 Lyon, France; alexandre.dufour3@univ-lyon1.fr (A.D.); f.mallein-gerin@ibcp.fr (F.M.-G.)

* Correspondence: emeline.groult@ibcp.fr

Abstract: Articular cartilage has limited potential for self-repair, and cell-based strategies combining scaffolds and chondrocytes are currently used to treat cartilage injuries. However, achieving a satisfying level of cell redifferentiation following expansion remains challenging. Hydrogels and perfusion bioreactors are known to exert beneficial cues on chondrocytes; however, the effect of a combined approach on the quality of cartilage matrix deposited by cells is not fully understood. Here, we combined soluble factors (BMP-2, Insulin, and Triiodothyronine, that is, BIT), fibrin hydrogel, direct perfusion and human articular chondrocytes (HACs) to engineer large cartilage tissues. Following cell expansion, cells were embedded in fibrin gels and cultivated under either static or perfusion conditions. The nature of the matrix synthesized was assessed by Western blotting and immunohistochemistry. The stability of cartilage grafts and integration with native tissue were also investigated by subcutaneous implantation of human osteochondral cylinders in nude mice. Perfusion preconditioning improved matrix quality and spatial distribution. Specifically, perfusion preconditioning resulted in a matrix rich in type II collagen but not in type I collagen, indicating the reconstruction of hyaline cartilage. Remarkably, the production of type VI collagen, the main component of the pericellular matrix, was also increased, indicating that chondrocytes were connecting to the hyaline matrix they produced.

Citation: Dufour, A.; Mallein-Gerin, F.; Perrier-Groult, E. Direct Perfusion Improves Redifferentiation of Human Chondrocytes in Fibrin Hydrogel with the Deposition of Cartilage Pericellular Matrix. *Appl. Sci.* **2021**, *11*, 8923. <https://doi.org/10.3390/app11198923>

Keywords: chondrocyte; cartilage tissue engineering; perfusion bioreactor; fibrin; type VI collagen

Academic Editor: Ming Pei

Received: 28 July 2021

Accepted: 23 September 2021

Published: 24 September 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Articular cartilage is a hyaline type of cartilage covering the extremities of long bones in synovial joints. The unique ability of this tissue to repeatedly absorb and transmit the load applied to the joint is due to the specific composition and structural organization of its extracellular matrix (ECM). Cartilage ECM is composed of proteoglycans, water and a collagen network, where type II collagen is the most abundant [1]. However, the avascular nature of cartilage together with the low metabolic rate of the chondrocytes limit its intrinsic capability for self-repairing [2]. Therefore, effective therapies for the treatment of articular cartilage lesions rely on surgical intervention and engraftments with a composition comparable to articular cartilage.

Current strategies for repairing articular cartilage include microfracture (i.e., marrow stimulation), mosaicplasty (i.e., osteochondral autograft), and autologous chondrocyte implantation (ACI) [3]. Microfracture and mosaicplasty remain unsatisfactory treatments due to the occurrence of type I collagen-rich fibrocartilage in the repaired defect [4,5]. ACI is a cell-based surgical procedure currently practiced as a popular second-line treatment for relatively large articular cartilage lesions [6,7]. However, the loss of therapeutic chondrocytes due to the liquid nature of the implant severely affects the quality of the healed defect [8,9]. As a consequence, scaffold-based approaches have been investigated

to guide matrix synthesis and deliver chondrocytes to the cartilage defect site. Hydrogels are excellent candidates for this due to their ability to encapsulate the cells, along with a high intrinsic water content (60–90%) [10], a feature found in native cartilage [11] which offers the tissue its “shock absorber” capabilities.

Nutrient availability to cells throughout three-dimensional (3D) scaffolds occurs in vitro by diffusion, and therefore depends on the distance from the surface, diffusional constraints, and cellular utilization at the periphery [12]. These diffusional limitations become exacerbated when attempting to engineer larger cartilage tissues. Perfusion bioreactors have been shown to enhance cell access to oxygen and nutrients as well as the homogeneity of neo-synthesized ECM in 3D scaffolds [13,14]; however, understanding of its impact on the quality of the ECM deposited by chondrocytes encapsulated in hydrogels remains limited [15–19].

Following this view, this work aimed to evaluate whether direct perfusion preconditioning could enhance cartilage matrix deposition by human articular chondrocytes (HACs) in fibrin hydrogel, a clinically relevant material that has been shown to support and maintain chondrocyte redifferentiation [20,21]. We used cocktails of specific factors to expand the cells on plastic (fibroblast growth factor (FGF)-2 and insulin, designated FI) and to allow their redifferentiation in fibrin hydrogel (bone morphogenetic protein (BMP)-2, insulin, and triiodothyronine T3, designated BIT). This sequential addition of factors has been proven efficient to amplify human chondrocytes isolated from diverse anatomical sites (including articular cartilage) and to induce their redifferentiation in collagen [22], agarose [23], self-assembling peptide [24,25] gels and collagen sponges [26,27]. Special attention was given to the quality and spatial distribution of the neosynthesized matrix through analysis of the pericellular matrix (PCM) components.

2. Materials and Methods

2.1. Cell Isolation and Expansion

HACs were isolated from macroscopically non-fibrillated zones of osteoarthritic joints from donors undergoing total or partial knee joint replacement (age range: 60–90; Ethics Committee for research with human samples, CODECOH: DC-2014-2325). Cartilage samples were sliced into small pieces (2 mm³) and digested overnight at 37 °C in culture medium composed of DMEM/F-12 (Invitrogen) with 0.5 mg/mL collagenase A (Roche Applied Science). The cell suspension was filtered, and isolated chondrocytes were seeded at a density of 1.5×10^4 cells/cm² in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen) and 50 µg/mL streptomycin (Panpharma) at 37 °C in 5% CO₂. When 48 h had elapsed post-seeding, the medium was replaced and supplemented with 5 ng/mL fibroblast growing factor-2 (FGF-2) (R&D Systems) and 5 µg/mL insulin (Umuline Rapide, Lilly). The culture medium was then replaced twice per week for two weeks.

2.2. Preparation of Fibrin Hydrogels

Human fibrinogen (Millipore) was dissolved in 10 mM HEPES (ThermoFisher Scientific) (pH 7.4) to obtain a 50 mg/mL fibrin solution. Likewise, human thrombin (Millipore) was dissolved in 10 mM HEPES (pH 6.5) supplemented with 0.1% BSA to prepare a 2 U/mL thrombin solution. A solution containing 3 M sodium chloride (NaCl) and 0.4 M calcium chloride (CaCl₂) was also prepared in 10 mM HEPES (pH 7.4). A 1.6 mL chondrocyte-fibrin suspension was then prepared by mixing 3.2×10^6 cells laden in 880 µL DMEM/F-12 with 320 µL fibrinogen, 320 µL thrombin and 80 µL NaCl/CaCl₂, yielding a final concentration of 10 mg/mL fibrinogen, 0.4 U/mL thrombin, 150 mM NaCl, 20 mM CaCl₂ and 2×10^6 cells/mL. In parallel, 2.5% low melting agarose (*w/v*) (Seaplaque, Cambrex BioScience) was poured in a petri dish and punched with a 10 mm biopsy punch to create cylindrical molds in which 314 µL of the chondrocyte/fibrin mixture was poured.

The cell-fibrin mixture was then allowed to gel at 37 °C in 5% CO₂ for 10 min to obtain cylindrical constructs of 10 mm diameter and 4 mm thick.

2.3. Static Culture

HAC-seeded fibrin scaffolds were placed in a 24-well plate and fresh chondrogenic medium containing 50 µg/mL 2-phospho-L-ascorbic acid, 200 ng/mL recombinant human BMP-2 (diboterminal- α , drug form of BMP-2, InductOs kit, Wyeth, Madison, USA), 5 µg/mL insulin and 100 nM triiodothyronine T3 (Sigma Aldrich, Saint Quentin Fallavier, France), that is, the BIT cocktail [27], was added. Medium was replaced twice a week over a culture period of 21 days.

2.4. Perfusion Culture

For the direct perfusion of cell-fibrin scaffolds, a bioreactor commercialized by Celtec Biotech AG (Basel, Switzerland) was used. The scaffold was placed between two silicon O-rings whose dimensions were similar to the hydrogel (10 mm diameter and 4 mm thick) (Figure 1). A 10 mm diameter synthetic mesh was placed within each silicon O-ring to hold the hydrogel in place and prevent any exit outside the culture chamber due to fluid flow. The gel entrapped between silicon holders and synthetic meshes was then placed in the polycarbonate culture chamber attached to two gas-permeable silicone columns and the chondrogenic medium was injected in the bioreactor. Finally, a constant culture medium flow rate of 2.5 µL/sec which did not affect cell viability (Figure S1) was established by a syringe pump (Programmable Harvard Apparatus PHD ULTRATM 2000). As for static culture, the medium was replaced twice a week over a 21 day culture period.

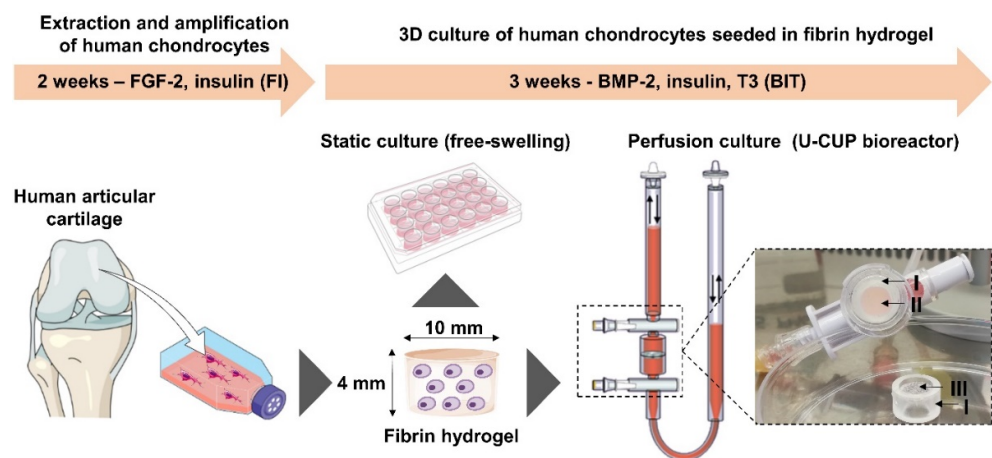


Figure 1. Scheme of the in vitro study and perfusion chamber. HACs were extracted from macroscopically healthy zones of knee cartilage and amplified for 2 weeks in monolayer with FGF-2 and insulin (FI) in the presence of 10% FBS. Cells were then seeded in fibrin hydrogel and cultured in the presence of BMP-2, insulin and T3 (BIT) in a well plate (static control) or a bidirectional perfusion bioreactor for 3 weeks. The perfusion chamber of the bioreactor was composed of (I) two silicone adaptors holding (II) the cartilage construct placed in between (III) two synthetic meshes.

2.5. In Vivo Subcutaneous Implantation

After 21 days of in vitro culture with perfusion, chondrocyte-fibrin constructs were fixed in osteochondral blocks (OC) and subcutaneously implanted in the back of nude mice to assess the maintenance of the redifferentiated phenotype of HACs, the stability of the neo-synthesized matrix, and the integration to native articular cartilage as previously reported with collagen sponge [27]. Human osteochondral blocks were created using an 8 mm diameter hollow punch (reference 694-C-320-N, SAM Outillage, Saint-Étienne, France). Defects 4 mm in diameter were then created at the surface of the OC blocks.

Following in vitro cultivation with perfusion for 21 days, cell-fibrin constructs were harvested on the edge with the same 4 mm diameter punching device and fixed into the defects using fibrin glue (Tisseel kit, Baxter, Meyzieu, France).

The animal experimentation was performed in strict accordance with the official regulation on animal experimentation (Directive 2010/63/EU and its national transposition) and ethical guidelines for care and use of mice of the Plateau de Biologie Expérimentale de la Souris (PBES, UMS 3444) at Ecole Normale Supérieure (ENS, Lyon, France). The in vivo study was approved by the Committee on the Ethics of Animal Experiments of ENS de Lyon (approval number: ENS_2018_007). Female nude mice (six weeks old) were obtained from Charles River Laboratories and maintained in the Animal Care Facilities of PBES under pathogen-free conditions with food and water *ad libitum*. The animals were regularly monitored by staff responsible for general animal health and welfare supervision. All surgeries were performed under general anesthesia and all efforts were made to minimize suffering. Animals were anesthetized with isoflurane gas, and surgeries were performed under a laminar flow hood in sterile conditions. A subcutaneous pocket was created on the dorsum of each mouse by blunt dissection through cranial and caudal skin incisions. The osteochondral block was inserted in the pocket and the skin was closed with surgical staples (Harvard Apparatus, ref 52-3746). After six weeks, the mice were euthanized by cervical dislocation. At explantation, all blocks were dissected from the mice and processed for histology.

2.6. Western Blot Analysis

After 21 days of in vitro culture, cell-fibrin hydrogels were frozen in liquid nitrogen. Without thawing, scaffolds were crushed with a mortar and pestle, and boiled in 2X Laemmli buffer containing 3% β -mercaptoethanol. 20 μ L of total protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4–15% Mini-PROTEAN® TGX™ gradient gels (Biorad, Marnes-la-Coquette, France). Proteins were transferred to an Immobilon PVDF membrane (Millipore, Molsheim, France) and probed with specific primary antibodies (Table 1) overnight at 4 °C. Membranes were then washed and incubated with secondary antibodies (Table 1). After several washes, bound antibodies were detected on X-ray films with Immun-star AP chemiluminescent substrate (Bio-Rad, Marnes-la-Coquette, France). When re-probed with antibodies, membranes were preliminary stripped with ReBlot Plus Strong solution (Millipore, Molsheim, France).

Table 1. Primary and Secondary Antibodies.

	Target Protein	Antibodies	Dilution	Source
Primary Antibodies	Type I collagen	Polyclonal anti-type I collagen	IB: 1:3000 IH: 1:2000	Novotec (Ref. 2941)
	Type II collagen	Polyclonal anti-type II collagen	IB: 1:2500 IH: 1:700	Novotec (Ref. 370j)
	Type VI collagen	Polyclonal anti-type VI collagen	IF: 1:200	Novotec (Ref. 20611)
	Type IX collagen	Monoclonal anti-type IX collagen (Clone 23-5D1)	IB: 1:3000	Millipore (Ref. MAB3304)
	Sox9	Polyclonal anti-Sox9	IB: 1:3000	Millipore (Ref. AB5535)
	Aggrecan	Polyclonal anti-Aggrecan core protein	IF: 1:250	Novotec (ref. CSPG CgEK 16.04.14)
	Actin	Polyclonal anti-Actin	IB: 1:2000	Sigma (Ref. A2066)
Secondary Antibodies		IgG AP-conjugated anti-Rabbit	1:3000	Cell Signaling Technology (Ref. 05/2016)
		IgG HRP-conjugated anti-Mouse	1:3000	Cell Signaling Technology (Ref. 11/2010)
		IgG HRP-conjugated anti-Mouse	Undiluted	Dako (Ref. K4002)
		IgG HRP-conjugated anti-Rabbit	Undiluted	Dako (Ref. K4002)
		Alexa Fluor 488-conjugated anti-Rabbit	1:500	Invitrogen (Ref. A11034)

IF, immunofluorescence; IH, immunohistochemistry; IB, Immunoblotting.

2.7. Histological and Immunohistochemical Analysis

Hydrogel constructs cultured *in vitro* for 21 days were fixed for 24 h in formol acetic alcohol (AFA, Microm Microtech, Brignais, France), then dehydrated in series of graded ethanol baths and embedded in paraffin. Osteochondral blocks subcutaneously implanted for six weeks in nude mice were first fixed in AFA for 24 h and then decalcified (ROD, Rapid Décalcifier) for 24 h. Following decalcification, blocks were fixed again in AFA for 24 h, then dehydrated and embedded in paraffin. Once in paraffin, *in vitro* and *in vivo* samples were cut in two before inclusion to observe the center on histological sections.

Histological stainings, immunohistochemistry (IHC), and immunofluorescence (IF) were performed on 5 µm sections. Deparaffinized sections stained with 0.04% (*w/v*) aqueous fast green solution for 4 min were then stained for 5 min in 0.2% (*w/v*) safranin-O solution for detection of proteoglycans. Deparaffinized sections were also stained with hematoxylin and eosin (HE). IHC was performed as previously described [26] with the relevant antibodies (Table 1). For IF, deparaffinized and rehydrated sections were incubated with 800 U/mL type IS hyaluronidase (Sigma Aldrich, Saint Quentin Fallavier, France) for 1 h at room temperature (RT) followed by cell permeabilization with 0.1% triton (Triton X-100, Sigma) for 10 min at RT. The sections were washed in phosphate-buffered saline (PBS) (Sigma Aldrich, Saint Quentin Fallavier, France) then incubated for 45 min at RT in 1% bovine serum albumin (BSA, Sigma Aldrich, Saint Quentin Fallavier, France) in PBS (BSA/PBS), followed by incubation with primary antibodies (Table 1) diluted in BSA/PBS overnight at 4 °C. The sections were then washed several times with PBS and incubated with secondary antibodies (Table 1) diluted in BSA/PBS for 1 h at RT. Nuclei were stained with 1X Hoechst (Fluka) in PBS for 5 min at RT. Following several PBS washes, the sections were mounted in 50% glycerol solution for observation.

2.8. Image Analysis

Samples were imaged with the Axioscan digital slide scanner (Carl Zeiss microscopy, GmbH, Marly Le Roi, France) and analyzed with ZEN 2 (blue edition) software (Carl Zeiss microscopy, GmbH). For safranin-O positive surface area measurements, Tagged Image File Format (TIFF) images were segmented in ImageJ software (version 1.15w, National Institutes of Health) with the Color Threshold plugin [28]. Safranin-O positive areas were isolated by setting Thresholding method to Default, Threshold color to black and white (B&W), Color space to HSB (Hue: 229/255, Saturation: 35/255, Brightness: 0/255) and Dark Background was not selected. The same parameters were applied to the total surface area of the histological section except for HSB sub-settings (Hue: 0/255, Saturation: 0/255, Brightness: 0/220). The value obtained was defined as a percentage by multiplying the ratio of safranin-O positive area on the total section area by 100. Measurements were performed on at least three entire histological sections per condition and per donor.

2.9. Statistical Analysis

Quantitative differences in protein synthesis and percentage of safranin-O positive area between experimental groups were analyzed using the non-parametric Mann–Whitney U test performed with Prism 6 software (version 6.01, GraphPad Prism Software, Inc., San Diego, USA).

3. Results

3.1. Spatial Distribution and Characterization of the Neo-Synthesized Matrix

We first evaluated the distribution of the neo-synthesized cartilage ECM produced by HACs in fibrin hydrogels. Following a 21 days culture period in the presence of BIT, static and perfused constructs were stained with fast green and counterstained with safranin-O to detect the presence of glycosaminoglycans (GAGs) of sulfated proteoglycans (Figure 2A). The presence of GAGs was detected in the outer periphery of the static gels, whereas sections of perfused gels showed a greater distribution of GAGs

through the construct, as confirmed with the isolation of safranin-O positive area with ImageJ software (Figure 2B). Quantification of the safranin-O positive area in the histological sections (Figure 2C) showed a significant difference between static and perfused gels where GAG positive area covered $3.6 \pm 5.5\%$ and $58 \pm 17\%$ (median \pm SD) of the section respectively.

High magnification of the periphery and core of the hydrogels confirmed GAG deposition only at the very periphery of the static gels, the intensity of the safranin-O staining rapidly decreasing towards the core where no GAG deposition was observed (Figure 2D). An intense and homogeneous safranin-O staining was found at the periphery of perfused gels whereas the core exhibited a lower staining intensity associated with strong ectopic staining. Thus, our observations clearly showed that perfusion resulted in superior accumulation and homogenous distribution of GAGs compared to static conditions. Similar observations were made for type II collagen, although mainly located around the cells with a limited diffusion in the extracellular space. Of note, intense ectopic accumulations of type II collagen were found in the core of the perfused gels. In contrast, immunostaining for type I collagen appeared similarly weak in static and perfused gels.

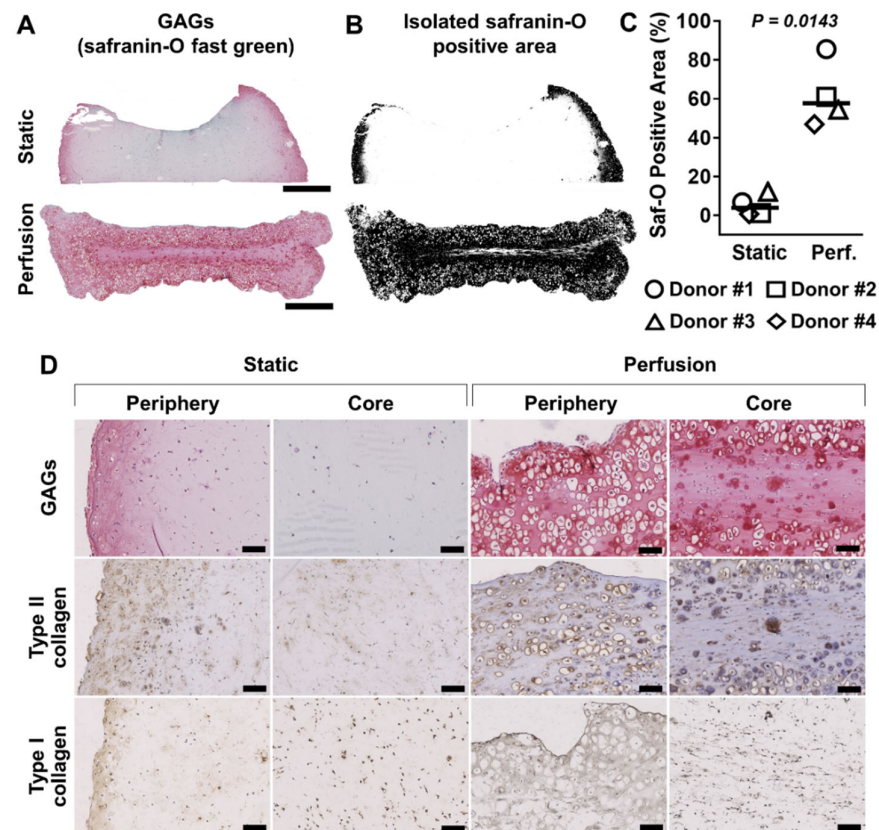


Figure 2. Perfusion enhances cartilage matrix deposition by HACs in fibrin hydrogel. HACs seeded in fibrin hydrogel were cultured in a well plate (static) or a direct perfusion bioreactor in the presence of BIT for 3 weeks. (A): Static and perfusion cultures stained for safranin-O fast green to reveal the presence of glycosaminoglycans (GAGs) of sulfated proteoglycans. (B): Isolation of safranin-O positive area (orange to red) within the histological sample with the color threshold plugin of ImageJ software. (C): Safranin-O positive surface area normalized to the total surface area of the histological section. Each single data point in the dot plot is the mean value of several histological slices; bars represent the median of the data points. (D): Representative images of safranin-O fast green staining (GAGs) and collagen types I and II immunohistochemical staining of the engineered cartilage constructs. The level of significance of the statistical analysis (*p*-value) is indicated (scale bars: A = 1 mm, D = 100 μ m) (*n* = 4).

In parallel cultures, we analyzed by Western blotting the proteins newly synthesized by the chondrocytes encapsulated within hydrogels. Type II and type IX collagen were detected in static and perfusion gels but perfusion significantly stimulated their synthesis ($p < 0.05$) (Figure 3A,B), with a relative fold change of 4 and 2.7, respectively. Type I collagen was produced to equivalent levels in the two culture conditions ($p > 0.1$, relative fold change of 1.3) (Figure 3A,B). Consequently, the type II/type I collagen ratio measured by densitometric analysis of the Western blots and taken as an index of chondrocyte differentiation was significantly higher in perfused gels than in static gels ($p < 0.05$) (Figure 3C). Of note, type II collagen was present as mature forms but also abundantly present as unprocessed pro-forms in all conditions, indicating that cells were still actively producing type II collagen after 21 days of culture. In parallel, the presence of type I collagen was restricted as mature forms, but not as total unprocessed chains (Figure 3A). This is a sign that HACs stopped their production of type I collagen and recovered their differentiated phenotype.

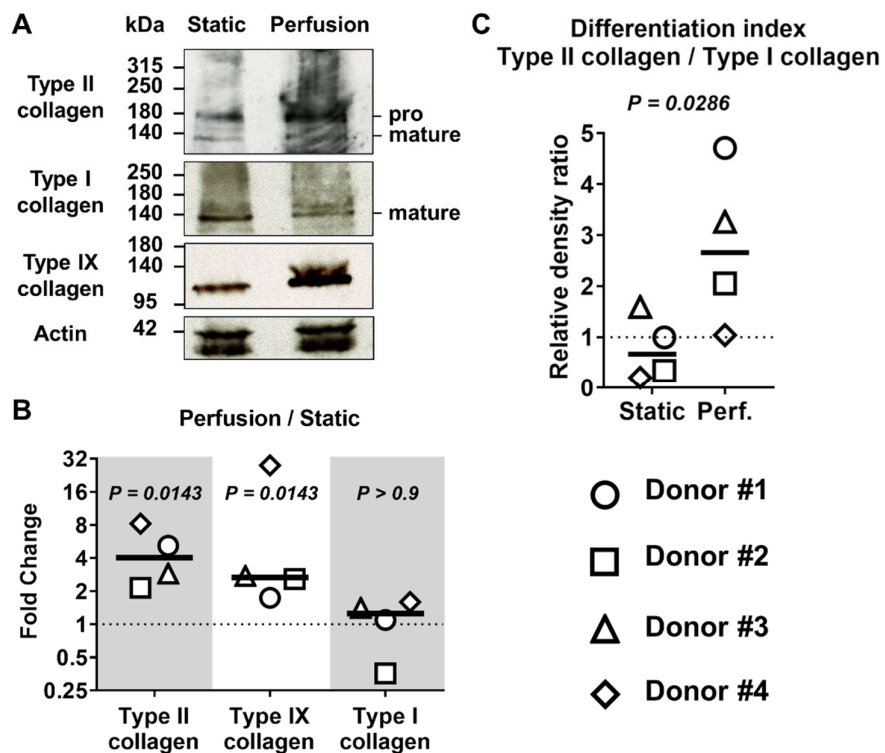


Figure 3. Perfusion promotes the synthesis of hyaline-type of cartilage. (A): Representative Western blot (WB) analysis of types I, II, and IX collagens. The positions of mature collagen chains (mature) and unprocessed (pro) are indicated. Actin bands are shown as a control for equivalent loading of the extracts. (B): Quantification of type II, type IX and type I collagen through the densitometric analysis of WB data. Total collagens (mature and unprocessed) were normalized to actin and the value of perfusion culture was then divided by the value obtained for the static culture of the same donor. (C): Ratio of densitometric values of type II collagen on type I collagen obtained in the same culture and defined as the differentiation index. Symbols in the dot plots represent a single donor data point and the bars represent the median. The level of significance of the statistical analyses (p -value) is indicated ($n = 4$).

3.2. HACs Remodel Their Environment and Self-Isolate in a Cartilage-Specific Pericellular Matrix

The pericellular environment of the chondrocyte plays a critical role in regulating cell activity because of the avascular nature of adult cartilage. Growth factors and matrix proteins passing to or from the cells may be retained in the PCM, where their structure or action may be further modified [29,30]. The PCM is rich in proteoglycans (e.g., aggrecan) and collagen types II, VI, and IX [31–33], but is typically defined by the exclusive presence and localization of type VI collagen around the chondrocyte as compared to the ECM [34,35]. We also investigated by immunofluorescence whether our cell culture conditions allowed chondrocytes to recapitulate the key components of the PCM (Figure 4). We first observed that type II collagen was present around the cells and diffused in the extracellular space, but was more abundantly produced when cells were cultured with fluid flow. We also observed a strong emission of immunofluorescence for aggrecan core protein around chondrocytes cultured with perfusion, such as in freshly isolated chondrons [32], whereas it was barely detectable in static cultures. Furthermore, a diffuse emission of immunofluorescence was also detected between cells, indicating a diffusion of the aggrecan core protein in the extracellular space. Lastly, the main component of the PCM, the type VI collagen, was clearly detected around chondrocytes cultured with perfusion, exhibiting the same specific localization as in the native tissue [34], while no evidence of its presence was detected in static gels. Of note and very interestingly, intracellular presence of aggrecan core protein and type VI collagen were found in perfused gels, indicating that cells were still active in the remodelling process of their pericellular environment.

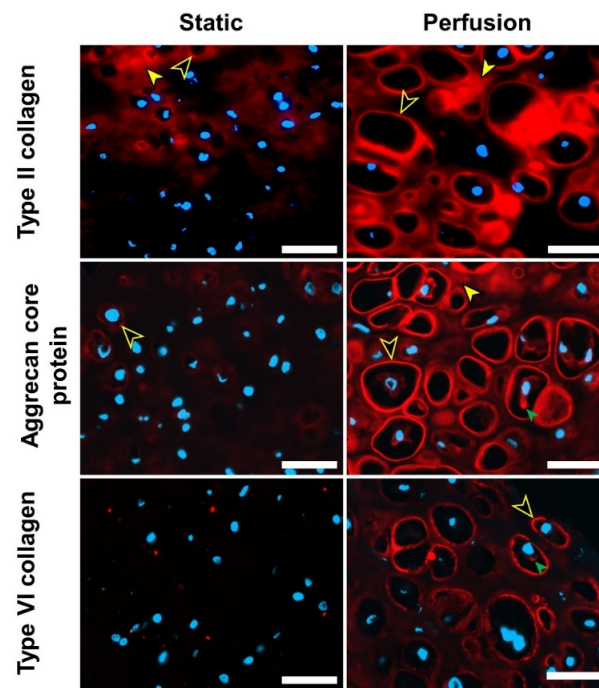


Figure 4. HACs self-isolate in a pericellular matrix similar to native cartilage when direct fluid-flow is applied. Representative immunofluorescence staining for type II collagen, aggrecan core protein, and type VI collagen. Proteins of interest were stained with the Alexa Fluor 488-conjugated secondary antibody (red). Nuclei were stained with Hoechst dye (blue). Arrows indicate pericellular immunostaining (black core and yellow border arrows), diffuse immunostaining in the extracellular space (yellow core and black border arrows) and intracellular immunostaining (small-sized green arrows) (scale bars = 50 μ m) ($n = 3$).

3.3. Perfusion Reinforces the Chondrogenic Activity through the Production of Sox9

To provide further evidence that perfusion of HACs encapsulated in fibrin hydrogel and treated with BIT enhances the chondrogenic activity of chondrocytes, we looked for Sox9 production. Sox9 is a major factor in developing and adult cartilage. It fulfils many important functions during chondrogenesis including transcriptional activation of many genes coding cartilage-specific structural components such as the type II and type IX collagen and aggrecan [36] studied here. Our Western blotting analysis confirmed that Sox9 was produced by HACs and perfusion stimulated its synthesis (Figure 5A,B), with a 2.4-fold relative change. We then looked more carefully at the cellular distribution of Sox9, depending on the culture condition. Close examination of Sox9 by immunofluorescence revealed an intense nuclear staining in both static and perfusion conditions (Figure 5C), probably as a consequence of the BIT treatment [24], which corresponds to a transcriptional function expected for active chondrocytes. These results together suggest that chondrocytes are functional in the two culture conditions, but the chondrogenic activity is more important when chondrocytes are cultured with perfusion.

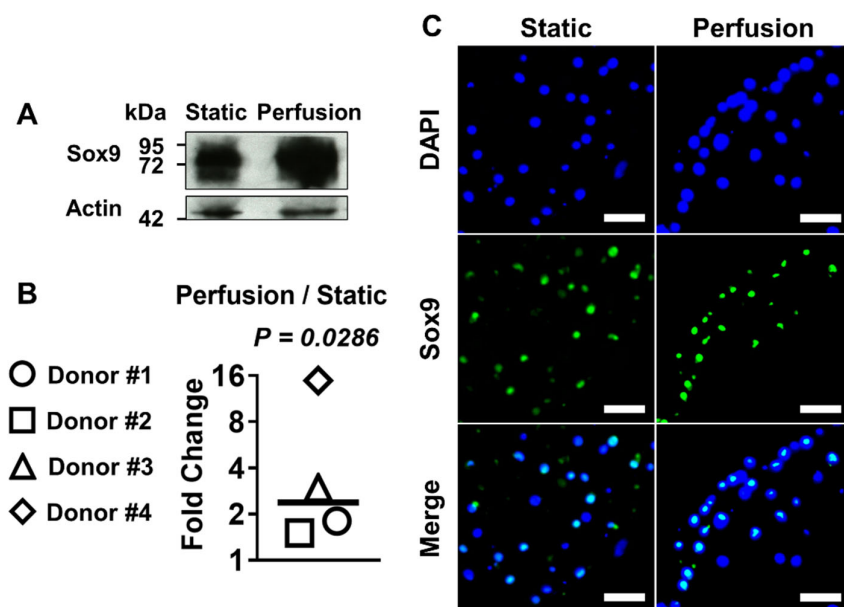


Figure 5. Perfusion increases Sox9 production by HACs. (A): Western blot (WB) analysis of Sox9 synthesis at the end of the in vitro culture period. Actin bands are shown as a control for equivalent loading of the extracts. (B): Quantification of Sox9 through the densitometric analysis of WB data. Sox9 was normalized to actin and the value of perfusion culture was then divided by the value obtained for the static culture of the same donor. Symbols in the dot plots represent a single donor data point and the bars represent the median. The level of significance of the statistical analysis (p -value) is indicated. (C): Immunofluorescence staining of HACs embedded in hydrogels. Cells were stained for Sox9 with the Alexa Fluor 488-conjugated secondary antibody (green). Nuclei were stained with Hoechst dye (blue) (scale bars = 50 μ m) ($n = 4$).

3.4. Stability of the Neo-Synthesized Matrix Following Subcutaneous Implantation

Finally, to evaluate the in vivo behaviour of redifferentiated HACs and the stability of the neo-synthesized matrix built under fluid pressure and in the presence of BIT, the constructs were glued into a cartilage defect made in a human osteochondral cylinder and subcutaneously implanted in nude mice (Figure 6).

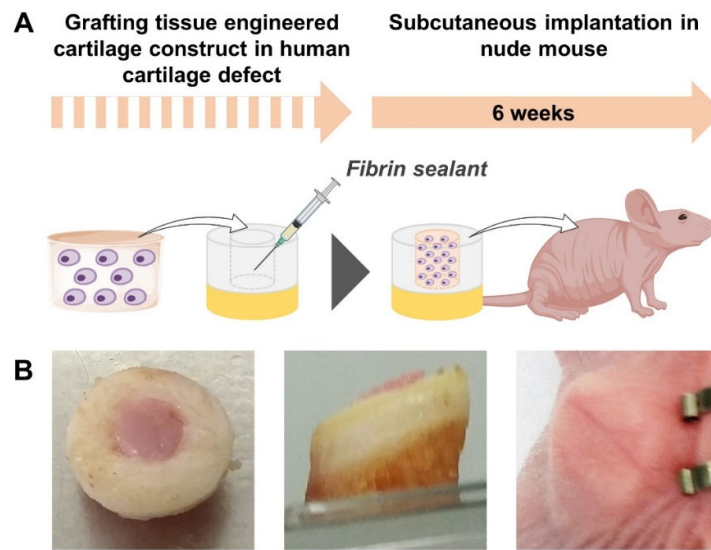


Figure 6. Scheme of the in vivo study. (A): Following extraction and monolayer expansion, HACs were seeded in fibrin hydrogel and cultured for 3 weeks in a direct perfusion bioreactor. Cartilage constructs were then fixed in fresh human osteochondral blocks with fibrin sealant and hybrid constructs were implanted into the subcutaneous pockets of nude mice for 6 weeks. The stability of the neo-synthesized matrix was assessed by histology. (B): Macroscopic pictures showing (left to right) the cartilage gel fixed in the human osteochondral block from top and side views as well as the hybrid construct implanted under mouse skin.

After 6 weeks in vivo, the constructs maintained their structural integrity and were processed for IHC analysis. HE staining (Figure 7A) showed the graft sitting at the junction between cartilage and bone, but poor integration was achieved with the surrounding native cartilage. IHC analyses revealed a strong accumulation of GAGs and type II collagen in the ECM (Figure 7B), whereas type I collagen was barely detected. Aggrecan core protein as well as type VI collagen were also detected in the implant (Figure 7C). These observations together demonstrate the persistence of the spatial distribution of PCM and ECM generated in vitro after six weeks of subcutaneous implantation in vivo and that chondrocytes were able to maintain their differentiated state after implantation.

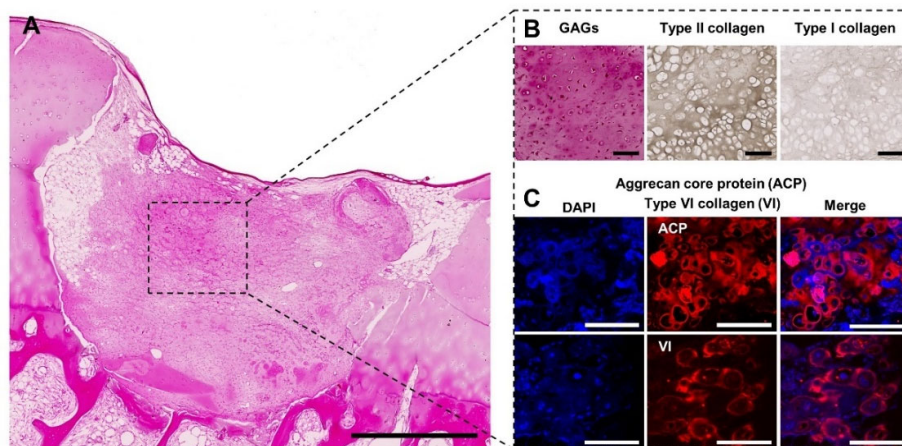


Figure 7. The cartilage construct remains stable after 6 weeks of subcutaneous implantation in nude mice. Following 21 days of culture in a bidirectional perfusion bioreactor in the presence of BIT, fibrin hydrogels seeded with HACs were placed into a cartilage defect made in human osteochondral blocks and then implanted into the subcutaneous pockets of

nude mice. (A): hematoxylin and eosin staining of the cartilage construct in the defect after 6 weeks of subcutaneous implantation. (B,C): Immunohistochemical analyses of the implant. (B): Cartilage implant stained for safranin-O fast green (GAGs) as well as for collagen types I and II. (C): Aggrecan core protein and type VI collagen immunofluorescence staining of the cartilage graft. Proteins of interest were stained with the Alexa Fluor 488-conjugated secondary antibody (red). Nuclei were stained with Hoechst dye (blue) (scale bars: A = 1 mm, B-C = 100 μ m).

4. Discussion

We investigated here the combined influence of a bidirectional perfusion bioreactor and an optimized sequential combination of soluble growth factors for the redifferentiation of HACs in fibrin hydrogel. The device we used in this study, namely the U-CUP bioreactor, is a commercially available perfusion bioreactor that takes into account important features of clinical translation, such as the ability to perform maturation of multiple constructs in individual chambers in parallel, automation and ease of use. Here, we applied a relatively low flow velocity based on previous studies. Freyria et al. defined an optimal flow rate between 1.6 and 5 μ L/s for direct perfusion of bovine chondrocytes seeded in collagen sponge and showed that a higher perfusion velocity (16 μ L/s) decreased GAG deposition [37]. Other studies have shown that a flow velocity of 0.13 or 8.3 μ L/sec increased GAG [15,16,18] and aggrecan [16] syntheses in agarose hydrogels by animal chondrocytes compared to static cultures. However, Grogan et al. reported that a perfusion velocity of 1.7 μ L/s reduced the production of GAGs and link protein by human chondrocytes in alginate hydrogels [17]. Here, our results demonstrate an increased production and improved quality of neo-synthesized cartilage matrix by HACs in fibrin hydrogels with low perfusion velocity (2.5 μ L/sec) compared to static cultures.

Perfusion preconditioning was applied to HAC-seeded gels of clinically relevant diameter, since 10 mm is the minimum defect diameter recommended for ACI [6,7] and is often reported as the average cartilage defect diameter in clinical studies involving ACI [38]. Perfusion clearly increased the quantity of cartilage ECM deposited in the large engineered tissues, with up to three quarters of the graft cross-section positively staining for GAG deposition. However, a delayed maturation of the core was indicated by ectopic cartilage ECM-positive areas, whereas the periphery of the gel seemed to have achieved its maturation as evidenced by homogeneous cartilage ECM deposition. This may be explained by an insufficient mass transfer of nutrients and growth factors to the core, as well as by lower fluid-induced shear stresses within the scaffolds which could have understimulated the cells.

In this study, we paid particular attention to the nature of the newly synthesized matrix. Our WB analyses showed that type II collagen, a typical marker of native cartilage [39], was produced both in static and in dynamic conditions. Undoubtedly, the combination of BIT and perfusion did enhance the deposition of type II collagen; it also enhanced that of type IX collagen, these being two main components of the collagen fibrils present in hyaline type of cartilage [40]. It is well-known that the amplification of HACs induces dedifferentiation, resulting in a progressive increase in type I collagen at the expense of type II collagen expression [41]. This “side effect” of HAC expansion is exacerbated with the FI cocktail despite its proliferative effect on HACs [27]. Thus, the chondrocytes were active in type I collagen production at the start of their culture in fibrine gel. However, no sign of precursor forms of type I collagen was detected, probably as a result of BIT treatment [24]. This stop in type I collagen production is particularly relevant since it indicates that the combination of fibrin and BIT favors the production of hyaline type cartilage as articular cartilage and not fibrocartilage (which has inferior mechanical properties). In a previous work, we showed that perfusion inhibited type I collagen production from HACs in collagen sponges [26]. Here, we observed such an effect only for one donor of four, type I collagen production being mainly equivalent between static and dynamic conditions. However, our differentiation index (type II collagen/type I collagen ratio) is in favor of a higher production of type II collagen and a differentiated phenotype in dynamic cultures. This is well supported by our histological

and immunohistochemical analyses showing the abundant presence of GAGs, type II collagen, aggrecan core protein, and type VI collagen, whereas type I collagen was barely detected in dynamic cultures.

The particular interest in type VI collagen in the current study arises from its unique structure and location. In articular cartilage, type VI collagen is almost exclusively located in the PCM. Its amino acid structure contains sequences that are active in binding to cellular receptors [42], and other sequences that are involved in the creation of multimeric fibrillar assemblies with collagen type II and aggrecan [43], which connect the chondrocytes to the macromolecular framework of the ECM. Such intimate interactions between the PCM components and the cell surface are likely involved in mechanotransduction to the cell surface; engineered constructs with non-native PCM structure could exhibit altered metabolic responses to physicochemical signals, affecting the ultimate success of such approaches. As a consequence, the presence of type VI collagen in our tissue-engineered constructs is an important indicator of advanced tissue maturation and demonstrates that cells emancipate themselves from the biomaterial to create intimate contacts with the matrix they produce. The absence of type VI collagen in static cultures also indicates that perfusion was the main determinant of the type VI collagen secretion observed in our study. These results together demonstrate that the combination of perfusion and BIT substantially improved the reconstruction of hyaline cartilage-specific ECM and PCM in fibrin hydrogels.

Perfusion can enhance the transport of molecules through large gels [15], but it also exerts mechanical forces such as fluid shear stress [44] and the expression of several ECM proteins, including type II and IX collagen and aggrecan, as well as transcription factors like Sox9, is sensitive to the mechanical forces in cartilage [45]. We also observed a statistically significant increase in the expression of type II collagen, type IX collagen, aggrecan, and Sox9 with direct perfusion (Figure S2). Thus, we can hypothesize that the fluid flow perfused here may have mechanically stimulated the chondrocytes seeded within fibrin gels. Importantly, the nuclear translocation of Sox9, the abundant expression of type II procollagen forms, and the presence of intracellular staining for aggrecan core protein and type VI collagen occurring in cells cultivated under direct perfusion attest that cells were active at the end of the culture period.

Although good stability of the neo-cartilage and maintenance of chondrocyte differentiated phenotype was observed after six weeks in the nude mouse model, pre-clinical trials with long-term implantation in cartilage lesions in a large animal model are required to assess the integration of the implant and mechanical stability in living joint. As to the latter, mechanical characterization of constructs was not performed here and this represents a limitation of the present study. Indeed, developing constructs with mechanical properties approaching those of native tissue is a key challenge in cartilage tissue engineering and the impact of perfusion preconditioning on the mechanical properties of the implant will be thus investigated in future studies.

Nevertheless, our results already demonstrate the value of a multi-factorial approach combining HACs from osteoarthritic joints, clinically relevant fibrin hydrogel, BIT cocktail, and perfusion preconditioning. This strategy leads to a high degree of cell redifferentiation by inducing the specific production and spatial distribution of hyaline matrix. This result is particularly relevant since it indicates that such a multi-factorial approach promotes the production of cartilage PCM, a key feature of functional cartilage. Of note, special efforts were made to develop an approach employing clinical-grade growth factors and scaffolds which, combined with the intrinsic features of the U-CUP bioreactor, can be easily translated into clinical practice. Lastly, since 3D bioprinting has the potential to modify the microarchitecture of large tissue-engineered constructs [46], it would be pertinent to investigate whether the introduction of microchannels within the graft or a positive gradient of fibrin concentration from the periphery to the core (i.e., leading to a progressive increase in microfiber spacing toward the core) could balance

access to growth factors and fluid-induced shear stresses between the center and the periphery of the graft.

5. Conclusion

The present study reports that perfusion preconditioning enhances redifferentiation of chondrocytes in fibrin hydrogel. Here, cells were extracted from human articular cartilage tissue, expanded, and cultured in fibrin gel. From a translational perspective, the approval by the Food and Drug Administration (FDA) of MACI[®] (Vericel, Cambridge, MA, USA)—where cells from the patient’s knee are expanded, placed on a scaffold, and then reimplanted into the patient—shows that tissue engineering strategies can be translated into clinical practice and are commercially feasible. Preconditioned grafts contained chondrocytes expressing a better level of redifferentiation at the gene and protein levels, adding potential therapeutic value. While this last point needs to be assessed in a large animal model, our results herein demonstrate that perfusion preconditioning of HACs in fibrin hydrogels generates cartilage-like tissues containing typical components of cartilage extra and pericellular matrix.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/app11198923/s1, Figure S1: cell viability in static and perfused gels after a 7 day culture period, Figure S2: Gene expression characterization of human articular chondrocytes cultivated for 21 days in fibrin hydrogel in free swelling (static) or a direct perfusion bioreactor, Figure S3: Quantification of aggrecan core protein and type VI collagen immunofluorescence at the single-cell level.

Author Contributions: Conceptualization, A.D. and E.P.-G.; methodology, A.D. and E.P.-G.; formal analysis, A.D.; data curation, A.D.; writing—original draft preparation, A.D.; writing—review and editing, A.D., F.M.-G. and E.P.-G.; supervision, F.M.-G. and E.P.-G.; project administration, F.M.-G. and E.P.-G.; funding acquisition, F.M.-G. and E.P.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CNRS, Lyon 1 University, and by Fondation de l’Avenir. A.D. was supported by a Ph.D. fellowship from the Région Auvergne-Rhône-Alpes (ARC2).

Institutional Review Board Statement: The study protocol involving human tissues was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Conservation D’Éléments du Corps Humain (CODECOH: DC-2014–2325) for preservation and research with human samples. The cartilage samples were collected after written informed consent of the donors. The study involving animals was conducted according to the guidelines of the Directive 2010/63/EU and its national transposition and ethical guidelines for care and use of mice of the Plateau de Biologie Expérimentale de la Souris (PBES, UMS 3444) at Ecole Normale Supérieure (ENS, Lyon) and approved by the Institutional Review Board of ENS Lyon (Committee on the Ethics of Animal Experiments, approval number: ENS_2018_007).

Informed Consent Statement: Informed consent was obtained from all human donors involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: Authors are grateful to the technical facilities of SFR Biosciences Gerland-Lyon (UMS3444/US8) for the quantitative PCR analyses and to EL KHOLTI Naima for her assistance with preparing and processing histological samples.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sophia Fox, A.J.; Bedi, A.; Rodeo, S.A. The basic science of articular cartilage: Structure, composition, and function. *Sports Health* **2009**, *1*, 461–468, doi:10.1177/1941738109350438.
2. Huey, D.J.; Hu, J.C.; Athanasiou, K.A. Unlike bone, cartilage regeneration remains elusive. *Science (80-)* **2012**, *338*, 917–921, doi:10.1126/science.1222454.

3. Richter, D.L.; Schenck, R.C.; Wascher, D.C.; Treme, G. Knee Articular Cartilage Repair and Restoration Techniques: A Review of the Literature. *Sports Health* **2016**, *8*, 153–160, doi:10.1177/1941738115611350.
4. Hangody, L.; Füles, P. Autologous osteochondral mosaicplasty for the treatment of full-thickness defects of weight-bearing joints: Ten years of experimental and clinical experience. *J. Bone Jt. Surg.-Ser. A* **2003**, *85*, 25–32, doi:10.2106/00004623-200300002-00004.
5. Hunziker, E.B. Articular cartilage repair: Basic science and clinical progress. A review of the current status and prospects. *Osteoarthr. Cartil.* **2002**, *10*, 432–463, doi:10.1053/joca.2002.0801.
6. Niemeyer, P.; Albrecht, D.; Andereya, S.; Angele, P.; Ateschrang, A.; Aurich, M.; Baumann, M.; Bosch, U.; Erggelet, C.; Fickert, S.; et al. Autologous chondrocyte implantation (ACI) for cartilage defects of the knee: A guideline by the working group “Clinical Tissue Regeneration” of the German Society of Orthopaedics and Trauma (DGOU). *Knee* **2016**, *23*, 426–435, doi:10.1016/j.knee.2016.02.001.
7. Makris, E.A.; Gomoll, A.H.; Malizos, K.N.; Hu, J.C.; Athanasiou, K.A. Repair and tissue engineering techniques for articular cartilage. *Nat. Rev. Rheumatol.* **2015**, *11*, 21–34, doi:10.1038/nrrheum.2014.157.
8. McCarthy, H.S.; Richardson, J.B.; Parker, J.C.E.; Roberts, S. Evaluating Joint Morbidity after Chondral Harvest for Autologous Chondrocyte Implantation (ACI): A Study of ACI-Treated Ankles and Hips with a Knee Chondral Harvest. *Cartilage* **2016**, *7*, 7–15, doi:10.1177/1947603515607963.
9. Sharpe, J.R.; Ahmed, S.U.; Fleetcroft, J.P.; Martin, R. The treatment of osteochondral lesions using a combination of autologous chondrocyte implantation and autograft. Three-year follow-up. *J. Bone Jt. Surg.-Ser. B* **2005**, *87*, 730–735, doi:10.1302/0301-620X.87B5.14936.
10. Zhao, W.; Jin, X.; Cong, Y.; Liu, Y.; Fu, J. Degradable natural polymer hydrogels for articular cartilage tissue engineering. *J. Chem. Technol. Biotechnol.* **2013**, *88*, 327–339, doi:10.1002/jctb.3970.
11. Flégeau, K.; Pace, R.; Gautier, H.; Rethore, G.; Guicheux, J.; Le Visage, C.; Weiss, P. Toward the development of biomimetic injectable and macroporous biohydrogels for regenerative medicine. *Adv. Colloid Interface Sci.* **2017**, *247*, 589–609, doi:10.1016/j.cis.2017.07.012.
12. Farrell, M.J.; Shin, J.I.; Smith, L.J.; Mauck, R.L. Functional consequences of glucose and oxygen deprivation on engineered mesenchymal stem cell-based cartilage constructs. *Osteoarthr. Cartil.* **2015**, *23*, 134–142, doi:10.1016/j.joca.2014.09.012.
13. Wendt, D.; Stroebel, S.; Jakob, M.; John, G.T.; Martin, I. Uniform tissues engineered by seeding and culturing cells in 3D scaffolds under perfusion at defined oxygen tensions. *Biorheology* **2006**, *43*, 481–488.
14. Santoro, R.; Olivares, A.L.; Brans, G.; Wirz, D.; Longinotti, C.; Lacroix, D.; Martin, I.; Wendt, D. Bioreactor based engineering of large-scale human cartilage grafts for joint resurfacing. *Biomaterials* **2010**, *31*, 8946–8952, doi:10.1016/j.biomaterials.2010.08.009.
15. Eniwumide, J.O.; Lee, D.A.; Bader, D.L. The development of a bioreactor to perfuse radially-confined hydrogel constructs: Design and characterization of mass transport properties. *Biorheology* **2009**, *46*, 417–437, doi:10.3233/BIR-2009-0552.
16. Schulz, R.M.; Wüstneck, N.; Van Donkelaar, C.C.; Shelton, J.C.; Bader, A. Development and validation of a novel bioreactor system for load- and perfusion-controlled tissue engineering of chondrocyte-constructs. *Biotechnol. Bioeng.* **2008**, *101*, 714–728, doi:10.1002/bit.21955.
17. Grogan, S.P.; Sovani, S.; Pauli, C.; Chen, J.; Hartmann, A.; Colwell, C.W.; Lotz, M.K.; D’Lima, D.D. Effects of perfusion and dynamic loading on human neocartilage formation in alginate hydrogels. *Tissue Eng.-Part A* **2012**, *18*, 1784–1792, doi:10.1089/ten.tea.2011.0506.
18. Nazempour, A.; Quisenberry, C.R.; Abu-Lail, N.I.; Van Wie, B.J. Combined effects of oscillating hydrostatic pressure, perfusion and encapsulation in a novel bioreactor for enhancing extracellular matrix synthesis by bovine chondrocytes. *Cell Tissue Res.* **2017**, *370*, 179–193, doi:10.1007/s00441-017-2651-7.
19. Yu, L.; Ferlin, K.M.; Nguyen, B.N.B.; Fisher, J.P. Tubular perfusion system for chondrocyte culture and superficial zone protein expression. *J. Biomed. Mater. Res.-Part A* **2015**, *103*, 1864–1874, doi:10.1002/jbm.a.35321.
20. Bianchi, V.J.; Lee, A.; Anderson, J.; Parreno, J.; Theodoropoulos, J.; Backstein, D.; Kandel, R. Redifferentiated Chondrocytes in Fibrin Gel for the Repair of Articular Cartilage Lesions. *Am. J. Sports Med.* **2019**, *47*, 2348–2359, doi:10.1177/0363546519857571.
21. Vogt, S.; Wexel, G.; Tischer, T.; Schillinger, U.; Ueblacker, P.; Wagner, B.; Hensler, D.; Wilisch, J.; Geis, C.; Wübbenhorst, D.; et al. The influence of the stable expression of BMP2 in fibrin clots on the remodelling and repair of osteochondral defects. *Biomaterials* **2009**, *30*, 2385–2392, doi:10.1016/j.biomaterials.2009.01.016.
22. Liu, G.; Kawaguchi, H.; Ogasawara, T.; Asawa, Y.; Kishimoto, J.; Takahashi, T.; Chung, U.I.; Yamaoka, H.; Asato, H.; Nakamura, K.; et al. Optimal combination of soluble factors for tissue engineering of permanent cartilage from cultured human chondrocytes. *J. Biol. Chem.* **2007**, *282*, 20407–20415, doi:10.1074/jbc.M608383200.
23. Durbec, M.; Mayer, N.; Vertu-Ciolino, D.; Disant, F.; Mallein-Gerin, F.; Perrier-Groult, E. [Reconstruction of nasal cartilage defects using a tissue engineering technique based on combination of high-density polyethylene and hydrogel]. *Pathol. Biol.* **2014**, *62*, 137–145, doi:10.1016/j.patbio.2014.03.001.
24. Dufour, A.; Buffier, M.; Vertu-Ciolino, D.; Disant, F.; Mallein-Gerin, F.; Perrier-Groult, E. Combination of bioactive factors and IEIK13 self-assembling peptide hydrogel promotes cartilage matrix production by human nasal chondrocytes. *J. Biomed. Mater. Res. Part A* **2019**, doi:10.1002/jbm.a.36612.
25. Dufour, A.; Lafont, J.E.; Buffier, M.; Verset, M.; Cohendet, A.; Contamin, H.; Confais, J.; Sankar, S.; Rioult, M.; Groult, E.P.; et al. Repair of full - thickness articular cartilage defects using IEIK13 self - assembling peptide hydrogel in a non-human primate model. *Sci. Rep.* **2021**, 1–17, doi:10.1038/s41598-021-83208-x.

26. Mayer, N.; Lopa, S.; Talò, G.; Lovati, A.B.; Padeloup, M.; Riboldi, S.A.; Moretti, M.; Mallein-Gerin, F. Interstitial perfusion culture with specific soluble factors inhibits type I collagen production from human osteoarthritic chondrocytes in clinical-grade collagen sponges. *PLoS ONE* **2016**, *11*, 1–18, doi:10.1371/journal.pone.0161479.
27. Claus, S.; Mayer, N.; Aubert-Foucher, E.; Chajra, H.; Perrier-Groult, E.; Lafont, J.; Piperno, M.; Damour, O.; Mallein-Gerin, F. Cartilage-Characteristic Matrix Reconstruction by Sequential Addition of Soluble Factors During Expansion of Human Articular Chondrocytes and Their Cultivation in Collagen Sponges. *Tissue Eng. Part C Methods* **2012**, *18*, 104–112, doi:10.1089/ten.tec.2011.0259.
28. Wang, J.H.; Lee, B.H. Mediolateral Differences of Proteoglycans Distribution at the ACL Tibial Footprint: Experimental Study of 16 Cadaveric Knees. *Biomed Res. Int.* **2018**, *2018*, doi:10.1155/2018/3762580.
29. Ruoslahti, E.; Yamaguchi, Y. Proteoglycans as modulators of growth factor activities. *Cell* **1991**, *64*, 867–869, doi:10.1016/0092-8674(91)90308-L.
30. Vincourt, J.B.; Etienne, S.; Grossin, L.; Cottet, J.; Bantsimba-Malanda, C.; Netter, P.; Mainard, D.; Libante, V.; Gillet, P.; Magdalou, J. Matrilin-3 switches from anti- to pro-anabolic upon integration to the extracellular matrix. *Matrix Biol.* **2012**, *31*, 290–298, doi:10.1016/j.matbio.2012.03.004.
31. Poole, C.A.; Honda, T.; Skinner, S.J.M.; Schofield, J.R.; Hyde, K.F.; Shinkai, H. Chondrons from articular cartilage (II): Analysis of the glycosaminoglycans in the cellular microenvironment of isolated canine chondrons. *Connect. Tissue Res.* **1990**, *24*, 319–330, doi:10.3109/03008209009152158.
32. Poole, C.A.; Glant, T.T.; Schofield, J.R. Chondrons from articular cartilage. (IV) Immunolocalization of proteoglycan epitopes in isolated canine tibial chondrons. *J. Histochem. Cytochem.* **1991**, *39*, 1175–1187, doi:10.1177/39.9.1717545.
33. Poole, C.A.; Gilbert, R.T.; Herbage, D.; Hartmann, D.J. Immunolocalization of type IX collagen in normal and spontaneously osteoarthritic canine tibial cartilage and isolated chondrons. *Osteoarthr. Cartil.* **1997**, *5*, 191–204, doi:10.1016/S1063-4584(97)80014-3.
34. Poole, C.A.; Ayad, S.; Schofield, J.R. Chondrons from articular cartilage: I. Immunolocalization of type VI collagen in the pericellular capsule of isolated canine tibial chondrons. *J. Cell Sci.* **1988**, *90 Pt 4*, 635–643.
35. Poole, C.A.; Flint, M.H.; Beaumont, B.W. Chondrons in cartilage: Ultrastructural analysis of the pericellular microenvironment in adult human articular cartilages. *J. Orthop. Res.* **1987**, *5*, 509–522, doi:10.1002/jor.1100050406.
36. Lefebvre, V.; Dvir-Ginzberg, M. SOX9 and the many facets of its regulation in the chondrocyte lineage. *Connect. Tissue Res.* **2017**, *58*, 2–14, doi:10.1080/03008207.2016.1183667.
37. Freyria, A.-M.; Yang, Y.; Chajra, H.; Rousseau, C.F.; Ronzière, M.-C.; Herbage, D.; Haj, A.J. El Optimization of Dynamic Culture Conditions: Effects on Biosynthetic Activities of Chondrocytes Grown in Collagen Sponges. *Tissue Eng.* **2005**, *11*, 674–684, doi:10.1089/ten.2005.11.674.
38. Na, Y.; Shi, Y.; Liu, W.; Jia, Y.; Kong, L.; Zhang, T.; Han, C.; Ren, Y. Is implantation of autologous chondrocytes superior to microfracture for articular-cartilage defects of the knee? A systematic review of 5-year follow-up data. *Int. J. Surg.* **2019**, *68*, 56–62, doi:10.1016/j.ijsu.2019.06.007.
39. Gouttenoire, J.; Valcourt, U.; Ronzière, M.C.; Aubert-Foucher, E.; Mallein-Gerin, F.; Herbage, D. Modulation of collagen synthesis in normal and osteoarthritic cartilage. *Biorheology* **2004**, *41*, 535–542.
40. Mendler, M.; Eich-Bender, S.G.; Vaughan, L.; Winterhalter, K.H.; Bruckner, P. Cartilage contains mixed fibrils of collagen types II, IX, and XI. *J. Cell Biol.* **1989**, *108*, 191–197, doi:10.1083/jcb.108.1.191.
41. Von Der Mark, K.; Gauss, V.; Von Der Mark, H.; Müller, P. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* **1977**, *267*, 531–532, doi:10.1038/267531a0.
42. Bonaldo, P.; Russo, V.; Bucciotti, F.; Doliana, R.; Colombatti, A. Structural and Functional Features of the $\alpha 3$ Chain Indicate a Bridging Role for Chicken Collagen VI in Connective Tissues. *Biochemistry* **1990**, *29*, 1245–1254, doi:10.1021/bi00457a021.
43. Wiberg, C.; Klatt, A.R.; Wagener, R.; Paulsson, M.; Bateman, J.F.; Heinegård, D.; Mörgelin, M. Complexes of matrilin-1 and biglycan or decorin connect collagen VI microfibrils to both collagen II and aggrecan. *J. Biol. Chem.* **2003**, *278*, 37698–37704, doi:10.1074/jbc.M304638200.
44. Moretti, M.; Freed, L.E.; Padera, R.F.; Laganà, K.; Boschetti, F.; Raimondi, M.T. An integrated experimental-computational approach for the study of engineered cartilage constructs subjected to combined regimens of hydrostatic pressure and interstitial perfusion. *Biomed. Mater. Eng.* **2008**, *18*, 273–278, doi:10.3233/BME-2008-0536.
45. Fitzgerald, J.B.; Jin, M.; Chai, D.H.; Siparsky, P.; Fanning, P.; Grodzinsky, A.J. Shear- and compression-induced chondrocyte transcription requires MAPK activation in cartilage explants. *J. Biol. Chem.* **2008**, *283*, 6735–6743, doi:10.1074/jbc.M708670200.
46. Murphy, S.V.; De Coppi, P.; Atala, A. Opportunities and challenges of translational 3D bioprinting. *Nat. Biomed. Eng.* **2020**, *4*, 370–380, doi:10.1038/s41551-019-0471-7.