

1 Regeneration of hyaline cartilage promoted by xenogeneic
2 mesenchymal stromal cells embedded within elastin-like
3 recombinamer-based bioactive hydrogels

4 David Pescador^{1,2}, Arturo Ibáñez-Fonseca^{3,4}, Fermín Sánchez-Guijo^{1,5}, Jesús G.
5 Briñón^{1,6}, Francisco Javier Arias³, Sandra Muntión^{1,5}, Cristina Hernández⁷, Alessandra
6 Girotti³, Matilde Alonso³, María Consuelo del Cañizo^{1,5}, José Carlos Rodríguez-
7 Cabello^{3*}, Juan Francisco Blanco^{1,2}

8 ¹ Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain

9 ² Servicio de Traumatología y Cirugía Ortopédica. Hospital Universitario de Salamanca,
10 Salamanca, Spain

11 ³ BIOFORGE Lab, Universidad de Valladolid, CIBER-BBN, Valladolid, Spain

12 ⁴ Technical Proteins Nanobiotechnology (TPNBT), Valladolid, Spain

13 ⁵ Unidad de Terapia Celular. Servicio de Hematología. Hospital Universitario de
14 Salamanca, Salamanca, Spain

15 ⁶ Departamento de Biología Celular y Patología. Universidad de Salamanca, Salamanca,
16 Spain

17 ⁷ Servicio de Radiodiagnóstico. Hospital Virgen de la Concha de Zamora, Zamora,
18 Spain

1 *Corresponding author:

2 Prof. J.C. Rodríguez-Cabello

3 roca@bioforge.uva.es

4 BIOFORGE (Group for Advanced Materials and Nanobiotechnology)

5 University of Valladolid

6 Edificio LUCIA

7 Paseo de Belén, 19

8 47011 – Valladolid

9 SPAIN

10 Running Title:

11 Osteochondral regeneration by hMSC-containing ELR-based hydrogels

12 Author Contributions:

13 FJA, FSG, MA, JCRC and JFB were involved in the conception and design of the study,

14 data analysis and interpretation, and in the critical revision for important intellectual

15 content. DP, AIF, AG, JGB and CH performed data acquisition, analysis and

16 interpretation, and were also involved in drafting the article or revising it critically for

17 important intellectual content. FSG, SM and MCdC collected and processed samples

1 from donors, while AG, FJA, MA and JCRC provided essential materials for the study.
2 All authors have read and approved the final submitted manuscript.

3 **ABSTRACT**

4 Over the last decades, novel therapeutic tools for osteochondral regeneration have arisen
5 from the combination of mesenchymal stromal cells (MSCs) and highly specialized
6 smart biomaterials, such as hydrogel-forming elastin-like recombinamers (ELRs), which
7 could serve as cell-carriers. Herein, we evaluate the delivery of xenogeneic human
8 MSCs (hMSCs) within an injectable ELR-based hydrogel carrier for osteochondral
9 regeneration in rabbits. First, a critical-size osteochondral defect was created in the
10 femora of the animals and subsequently filled with the ELR-based hydrogel alone or
11 with embedded hMSCs. Regeneration outcomes were evaluated after three months by
12 gross assessment, magnetic resonance imaging and computed tomography, showing
13 complete filling of the defect and the *de novo* formation of hyaline-like cartilage and
14 subchondral bone in the hMSC-treated knees. Furthermore, histological sectioning and
15 staining of every sample confirmed regeneration of the full cartilage thickness and early
16 subchondral bone repair, which was more similar to the native cartilage in the case of
17 the cell-loaded ELR-based hydrogel. Overall histological differences between the two
18 groups were assessed semi-quantitatively using the Wakitani scale and found to be
19 statistically significant ($p < 0.05$). Immunofluorescence against a human mitochondrial
20 antibody three months post-implantation showed that the hMSCs were integrated into
21 the *de novo* formed tissue, thus suggesting their ability to overcome the interspecies
22 barrier. Hence, we conclude that the use of xenogeneic MSCs embedded in an ELR-

1 based hydrogel leads to the successful regeneration of hyaline cartilage in osteochondral
2 lesions.

3 **KEYWORDS**

4 hyaline cartilage; human mesenchymal stromal cells; elastin-like recombinamers;
5 osteochondral regeneration; xenotransplantation

6

1 INTRODUCTION

2 Osteochondral injuries are a frequent source of pain and disability and can even result in
3 secondary osteoarthritis.^{1,2} This disease has an enormous economic impact in developed
4 countries, mostly due to ageing.^{3,4} One of the main issues in this regard is that articular
5 cartilage possesses limited regeneration ability due to its avascularity and the cellular
6 and interstitial structure that sustains the biomechanical requirements of joints.⁵ As
7 such, it is easy to understand the efforts of orthopedic surgeons in developing novel
8 therapies in this field.⁶

9 The development of novel tissue-engineering methods^{7,8} involving mesenchymal
10 stromal cell (MSC) therapy in combination with highly specialized biomaterials has
11 opened up a broad field for the study of articular cartilage regeneration.⁹ MSCs are
12 pluripotent cells that can easily be isolated and expanded *in vitro*. Furthermore, these
13 cells can differentiate into diverse cell types (including chondrocytes and osteocytes)
14 and exert immunomodulatory properties, which makes them good candidates for the
15 treatment of musculoskeletal lesions.^{10,11} There are different auto-, allo- and xenogeneic
16 sources of MSCs. However, whereas the first two options offer an immunologically
17 safer approach, the latter increases the availability of MSCs enormously. Indeed, there
18 are numerous studies describing the successful use of xenogeneic MSCs in different
19 hosts.¹² Since pre-differentiated cells have been proposed to induce immunological
20 reactions in xenotransplantation models,¹³ differentiation may be induced *in vivo* in the
21 presence of some specific endogenous factors in the niche where MSCs are
22 implanted.^{14,15}

1 In contrast, the application of a single suspension of MSCs may lead to poor retention
2 and viability of cells,^{16,17} therefore the use of scaffolds is highly recommended in order
3 to increase the persistence and engraftment of the implanted cells at the site of injury.
4 To this end, biomaterials that mimic the extracellular matrix (ECM) and acquire a 3D
5 structure that could be re-populated by cells might be very helpful. Such materials
6 should also be compatible with non-invasive techniques, as is the case for injectable
7 hydrogels. Over the last few decades recombinant DNA techniques have proven to be
8 very powerful tools for the development of novel protein biomaterials that are able to
9 self-assemble into different structures, such as hydrogels.^{18,19} These biomaterials
10 include elastin-like recombinamers (ELRs),²⁰ the composition of which is based on
11 repetition of the VPGXG pentapeptide found in natural elastin, in which X (guest
12 residue) can be any amino acid except L-proline. ELRs show thermosensitivity, which
13 is characterized by a temperature known as the transition temperature (Tt), above which
14 ELRs assemble hydrophobically, undergoing a phase transition, whereas they remain
15 soluble at lower temperatures,^{21,22} thus permitting a homogeneous embedding of MSCs.
16 These ELR molecules can self-assemble into hydrogels above the Tt, *i.e.*, at
17 physiological temperature, when designed with a very specific composition,²³ thus
18 allowing the use of the cell-scaffold system in arthroscopy or in injectable therapies,
19 where they adopt the shape of the injured tissue. As a result of their recombinant nature,
20 further genetic modification of these ELRs by addition of the well-known RGD cell-
21 adhesion sequence, which promotes specific cell attachment via integrins,²⁴ leads to an
22 injectable scaffold that provides a cell-friendly environment.

1 With regard to the tracking of cartilage regeneration, non-invasive imaging techniques
2 that allow patients to be monitored are highly encouraged. Magnetic resonance imaging
3 (MRI) is the most frequently used imaging method for the evaluation of chondral
4 injuries,²⁵⁻²⁷ and this technique is complemented with computed tomography (CT) when
5 subchondral bone has also been damaged.²⁸

6 Despite the ability to monitor evolution of the injured tissue *in vivo*, histology is still
7 essential to demonstrate the regenerative potential of novel treatments when tested in
8 animals. In fact, several methods, such as those of O'Driscoll or Wakitani, aim to
9 quantitatively assess articular cartilage repair by microscopic observation, thereby
10 proposing a more efficient approach for the comparison of different therapies.²⁹

11 In this work, we hypothesized that human MSCs (hMSCs) embedded in an ELR-based
12 hydrogel resembling the ECM would be able to regenerate an osteochondral critical-size
13 defect in a xenotransplantation model in rabbits. Herein, we show results from gross
14 assessment, imaging techniques (MRI and 3D CT) and microscopic evaluation using the
15 Wakitani histological scoring system.

16 **MATERIALS AND METHODS**

17 *2.1. Ethical approval*

18 All procedures regarding collection of hMSCs specified below were approved by the
19 Ethics Committee of the University Hospital of Salamanca (Spain) and were also in

1 accordance with the Declaration of Helsinki (1975), as revised in 2000. Informed
2 written consent was obtained from all subjects included in the study.

3 All animal experiments were conducted in accordance with the institutional guidelines
4 for the care and use of experimental animals of the University of Salamanca (Spain) in
5 accordance with Directive 2010/63/EU (Resolution Number 2010/2/23).

6 *2.2. Human mesenchymal stromal cell (hMSC) collection*

7 A volume of 5 mL of bone marrow was obtained from six healthy donors (mean age 55
8 years), by conventional iliac crest aspiration, at the University Hospital of Salamanca.
9 Low density mononuclear cells (MNCs) were isolated using a Ficoll-Paque gradient
10 (Biochrom KG, Berlin, Germany) and plated at a cell density of 10^6 MNC/cm² on a
11 polystyrene surface in DMEM (Gibco BRL, Paisley, UK) containing 5% platelet lysate,
12 obtained as reported previously.³⁰ Culture flasks were maintained in a humidified
13 incubator at 37 °C and 5% CO₂, with medium replacement twice a week, removing non-
14 adherent hematopoietic cells. The cell layer was trypsinized at 80% confluence and cells
15 were subcultured at a cell density of $2.5 \cdot 10^3$ MNC/cm². Methods and the results of
16 MSC characterization, according to previous studies, are summarized in the
17 Supplementary Material (Supplementary Methods).³¹⁻³³

18 *2.3. ELR design, bioproduction and characterization*

19 The elastin-like recombinamer (ELR) used in this work was genetically engineered as
20 described elsewhere¹⁹ and provided by Technical Proteins Nanobiotechnology (TPNBT)

1 S.L. (Spain). Briefly, the ELR was recombinantly bioproduced in *Escherichia coli* in a
2 15-L bioreactor (Applikon Biotechnology, Netherlands) and purified by several cooling
3 and heating purification cycles (Inverse Transition Cycling) following centrifugation.
4 The ELR sequence and its description can be found in the Supplementary Information
5 (Supplementary Methods and Table S-1).

6 In order to study the stability of the resulting hydrogels, they were formed by dissolving
7 the ELR in cold phosphate buffered saline (PBS, pH 7.4) at a concentration of 75
8 mg/mL in glass vials, and heated to 37 °C for 15 minutes. Another vial was kept at 4 °C
9 during that time. Both vials were then placed face down and photographs taken for
10 comparison.

11 *2.4. In vivo experimental model*

12 Six male New Zealand white rabbits with an age of 6 months and an average weight of
13 3.45 kg were used for the creation and treatment of the osteochondral defects. Animals
14 were anesthetized intramuscularly with xylazine (5 mg/kg) and ketamine (35 mg/kg),
15 then both knees were shaved and cleaned. A parapatellar incision of the skin was
16 performed under sterile conditions in order to expose the distal femur. A 4x4 mm full-
17 thickness critical-size osteochondral lesion was created with a drill. The defect was deep
18 enough to reach the osteochondral bone in every case. Immediately afterwards, a cold
19 solution containing $0.5 \cdot 10^6$ hMSCs embedded in 2 mL of the hydrogel solution (75
20 mg/mL, culture medium) was used to completely fill the defect of the right knee (n = 6),
21 whereas the hydrogel under the same conditions but without cells was placed in the left

1 knee to serve as control (n = 6). The wound was closed after less than 1 minute, and the
2 hydrogel fully solidified and adapted itself to the surface of the lesion, as confirmed by
3 visual observation, after coming into contact with the animal tissue, at a temperature
4 above the Tt of the ELR. Gentamicin (5 mg/kg) was administered after the surgical
5 procedure to avoid infection. All animals were fed and watered *ad libitum* during the
6 study period and maintained in individual cages.

7 Animals were euthanized with pentobarbital (120 mg/kg) at three months post-treatment
8 and the distal femora were extracted for further analysis.

9 *2.5. MRI and 3D CT*

10 Whole legs from two animals were used for image collection using a 1.5 Tesla MRI
11 instrument (Signa LX version 9.1, General Electric), with the sequences Oblique 3D
12 Fast Spoiled Gradient Echo (FRPGR) Special and 3D Spoiled Gradient (3D SPGR).

13 Two other specimens were used for extraction of both femoral condyles for further
14 image analysis using multi-slice helical CT (Aquilion 16, Toshiba, Japan), obtaining
15 axial sequences of 0.5 mm thickness on the bone window and multi-slice 3D
16 reconstructions in the sagittal and coronal planes.

17 *2.6. Histological analysis*

18 Extracted samples were fixed in 4% formaldehyde and 0.2% picric acid in PBS 0.1 M
19 (pH 7.3) at 4 °C. They were subsequently processed as described in the Supplementary
20 Information.

1 A blind macro- and microscopic analysis was performed on the samples by a trained
2 histologist. Samples from each rabbit (n = 6 for each group) were classified on the basis
3 of their modified Wakitani score³⁴ (Table S-2, maximum score of 15) and inter-group
4 comparisons were performed using the non-parametric Kruskal–Wallis test, with a p-
5 value < 0.05 indicating statistically significant differences.

6 To evaluate the survival of human MSCs, a mouse monoclonal antibody against a 65
7 kD mitochondrial membrane protein specifically expressed in human cells (MAB1273,
8 Millipore) was used, followed by a secondary antibody, namely donkey anti-mouse IgG,
9 conjugated with Cy3 (Jackson ImmunoResearch Europe, Ltd., U.K). The combination
10 with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to A-
11 T rich regions in DNA, allowed all nuclei from both human and rabbit cells to be
12 stained, and thus the xenogeneic cells in the regenerated tissue to be observed in
13 comparison with host cells.

14 Images were taken under similar conditions of exposure and time for every sample, as
15 stated in the Supplementary Information.

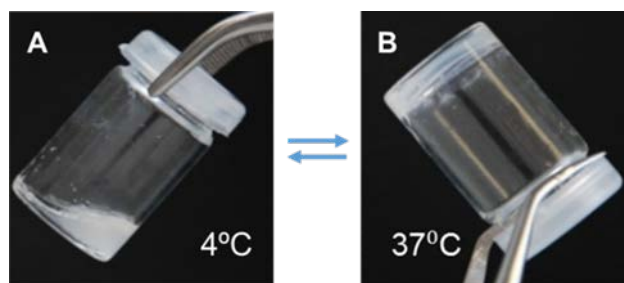
16 **RESULTS**

17 *3.1. ELR bioproduction and characterization*

18 The ELR was successfully bioproduced in *E. coli*, purified by ITC and stored
19 lyophilized at -20 °C until further use. The bioproduction yield was found to be an
20 average of 200 mg ELR/L of culture.

1 SDS-PAGE and MALDI-TOF confirmed the purity, integrity and molecular weight of
2 the ELR, which coincided with the theoretical value. Thus, the experimental molecular
3 weight was found to be 112,253 Da, while the theoretical value was 112,270 Da (Figure
4 S-1). Moreover, the Tt was found to be 15.3 °C in PBS (Figure S-2), which means that
5 hydrogels are formed at physiological temperature upon ELR transition. The ¹H NMR
6 spectrum (Figure S-3 and Table S-3) and amino acid analysis (Table S-4) showed the
7 absence of contaminants in the final ELR product.

8 To macroscopically assess hydrogel formation, the ELR was dissolved at 4 °C and then
9 warmed to 37 °C. The vial containing the hydrogel was then placed upside-down and
10 the hydrogel found to be formed and stable, in other words it did not fall from the
11 bottom of the tube or dilute in an aqueous solvent (Figure 1).



12

13 **Figure 1.** ELR dissolved at 4 °C (A) and hydrogel formed at 37 °C (B), inverted to show
14 the hydrogel state and its stability.

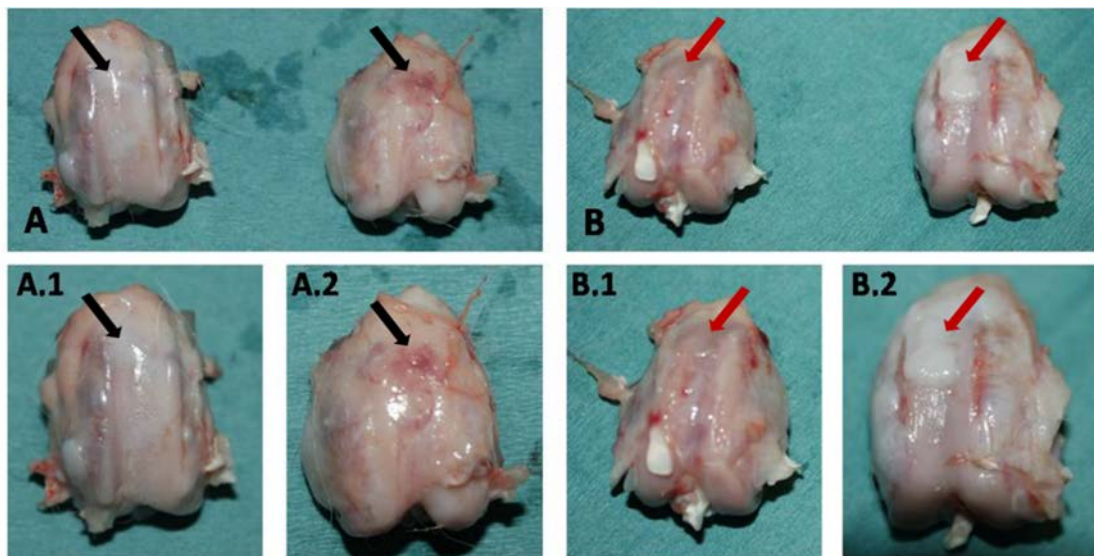
15 3.2. Macroscopic evaluation

16 A. Control group (ELR)

1 A partially regenerated whitish region could be observed macroscopically in the
2 extracted femora, with this area being paler than the surrounding normal cartilage.
3 Furthermore, the appearance thereof was not smooth and it presented a fibrotic surface,
4 with protrusions (Figure 2, red arrows) projecting slightly from the rest of the articular
5 surface (Figure 2, A2 and B2), thus differing from the appearance of non-injured
6 cartilage.

7 B. Experimental group (ELR + hMSCs)

8 Regenerated tissue with a smooth and bright appearance was observed in all cases. The
9 injured region could be recognized by its more greyish colour in comparison with the
10 regenerated cartilage. Moreover, the regenerated tissue was well integrated into the
11 surrounding chondral tissue, with continuity between the two structures (Figure 2, A1
12 and B1, black arrows), thus resembling the situation found in non-injured cartilage.



13

1 **Figure 2.** Images showing the comparison between femurs extracted from the same
2 animals (A and B) treated either with hMSCs combined with the ELR-based hydrogel
3 (experimental group) (A1, B1), or with the ELR-based hydrogel alone (control group)
4 (A2, B2). Defects are indicated with black and red arrows for the experimental and the
5 control group, respectively.

6 *3.3. Image analysis*

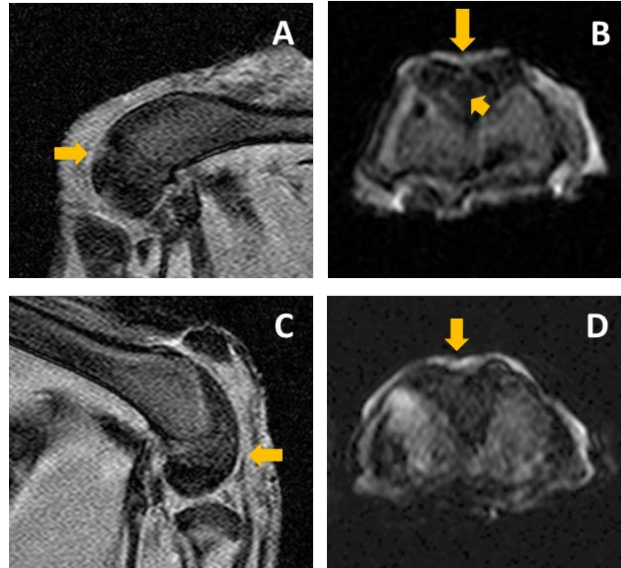
7 3.3.1. MRI

8 A. Control group (ELR)

9 The 3D SPGR (Figure 3A) magnetic resonance sequence showed articular cartilage loss
10 (yellow arrow) and an underlying bone oedema of approximately 3 mm along the major
11 axis in both samples studied. Similarly, axial 3D FSPGR (Figure 3B) showed a
12 discontinuity in the articular cartilage of the femoral condyle (long yellow arrow) with
13 underlying bone lesion (short yellow arrow).

14 B. Experimental group (ELR + hMSCs)

15 Regenerated articular cartilage could be observed in all sequences acquired, showing a
16 healthy subchondral bone (3D SPGR, Figure 3C, yellow arrow), while the condyle
17 surface showed no fractures or traces of bone oedema in any case (3D FSPGR, Figure
18 3D, yellow arrow highlighting regenerated defect), thus suggesting complete restoration
19 of the injured tissue in every sample.



1

2 **Figure 3.** MRI analysis of femurs extracted from rabbits in the control group (ELR
 3 hydrogel, A and B) and experimental group (ELR hydrogel + hMSCs, C and D). A and
 4 C, and B and D correspond to the 3D SPGR and 3D FSPGR sequences, respectively.

5 3.3.2. 3D CT

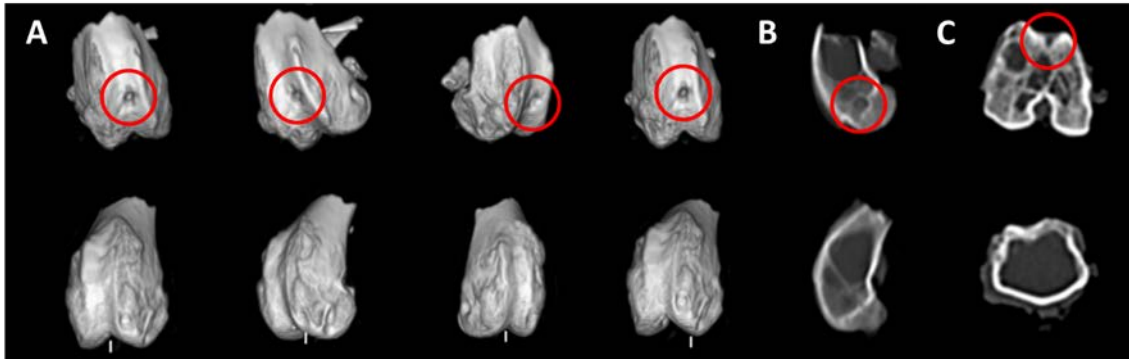
6 A. Control group (ELR)

7 A 3D reconstruction of the femoral condyles (Figure 4 (top)) showed a bone defect of
 8 up to 3x3 mm in the transverse and anteroposterior planes, with articular cartilage loss,
 9 thus indicating a partially repaired osteochondral lesion (red outlines). These findings
 10 were confirmed in the sagittal and axial plane images (Figure 4B and C, respectively).

11 B. Experimental group (ELR + hMSCs)

12 The osteochondral defect was imperceptible in the specimen from the experimental
 13 group (Figure 4, bottom), thus indicating an almost fully regenerated injury (Figure 4A).

1 Sagittal (Figure 4B) and axial (Figure 4C) plane images corroborated the result found in
2 the 3D reconstruction, thereby also confirming the MRI findings.



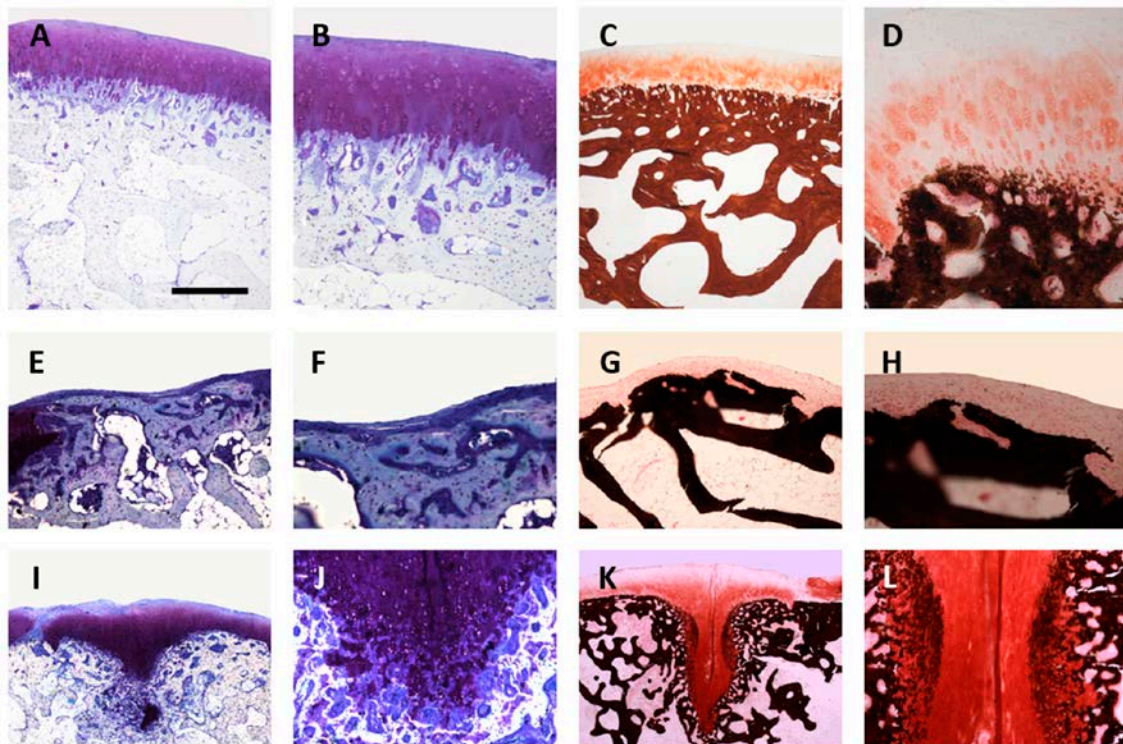
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4 **Figure 4.** 3D CT reconstructions (A) and sagittal and axial slices (B and C) of femurs
5 from rabbits in the control group (top) and experimental group (bottom). Marked
6 defects are highlighted in red.

7 *3.4. Histological analysis*

8 Toluidine blue stain was used to clarify the distribution of cells and collagen fibres in
9 the extracellular matrix, as well as cell morphology, thus allowing the observation of
10 osteoblasts and osteocytes, together with bone lamellas and bone marrow cells, the
11 cytoplasm of which was stained a deep blue colour. Furthermore, this metachromatic
12 colorant allowed the identification of newly formed bone tissue (stained violet), with the
13 chondral matrix being stained into reddish and purple hues. In addition, Von Kossa
14 staining allowed calcified regions to be differentiated from other tissue by its dark
15 brown colour.

1 3.4.1. Microscopic evaluation

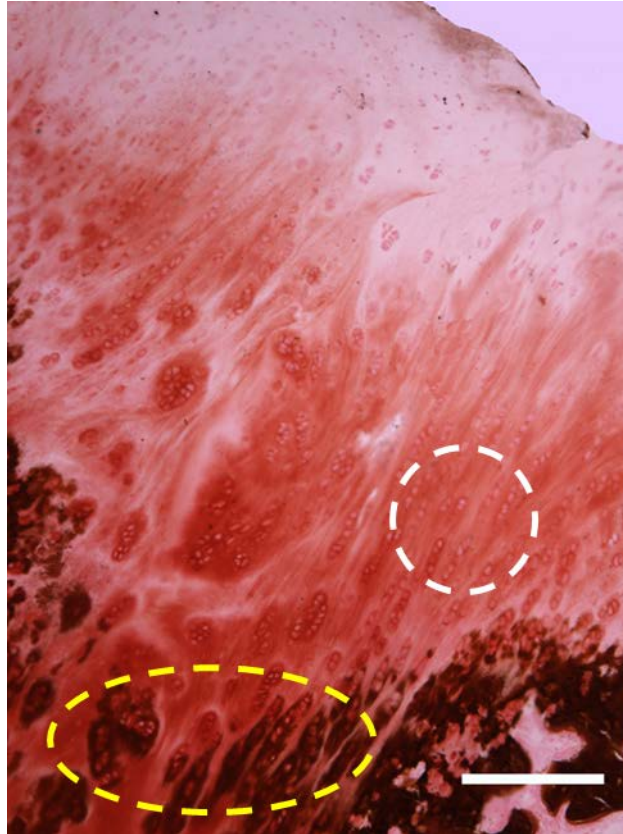
2 The regenerated tissue at the articular surface of samples from the control group
3 exhibited a minimal thickness in comparison with adjacent non-injured articular
4 cartilage (Figure 5). Moreover, this tissue showed a fibrotic appearance, similar to the
5 features of fibrocartilage, arranged on a base of subchondral bone trabeculae that is
6 mostly regenerated. This fibrocartilage-like tissue was composed of small egg-shaped
7 cells, the major axis of which was oriented parallel to the articular surface, following the
8 orientation of the fibres that compose the matrix in which the cells are embedded.
9 However, this tissue was not found to be integrated with the surrounding hyaline
10 cartilage present at the surface of non-injured cartilage.



11

1 **Figure 5.** Toluidine blue (left) and Von Kossa (right) staining of non-injured tissue (A-
2 D) and samples from the control (ELR, E-H) and experimental groups (ELR + hMSCs,
3 I-L). Scale bar corresponds to 1 mm for I and K, 500 μm for A and C, 250 μm for B, D,
4 E, G, J and L, and 125 μm for F and H.

5 In contrast, coronal sections from samples from the experimental group (ELR +
6 hMSCs) showed the osteochondral defect area to be entirely filled with a tissue
7 identified as hyaline cartilage. This regenerated cartilage displayed a smooth and regular
8 surface and was completely integrated with the adjacent non-injured cartilage, with no
9 signs of discontinuity in any case. The different layers comprising *de novo* formed
10 cartilage could also be clearly observed, showing no structural differences with respect
11 to healthy cartilage. The defect in the subchondral bone beneath the regenerated
12 articular cartilage was filled with a tissue with the structural and staining features of
13 cartilage. Regions of hypertrophied chondrocytes could be found towards the edges of
14 the cartilage near the subchondral bone, and the neighbouring matrix was moderately
15 calcified, which was identified as an endochondral ossification process. These areas
16 continued to the edges of the injured zone until they reached trabeculae from intact bone
17 tissue, with no interruption with the adjacent bone. Filling of cartilaginous tissue was
18 observed in the bone area of the lesion. A further demonstration of these results can be
19 seen in Figure 6, in which a well regenerated articular cartilage (white outline) can be
20 seen together with ossification areas below it (yellow outline), showing a similar
21 structure to that found in native osteochondral tissue.



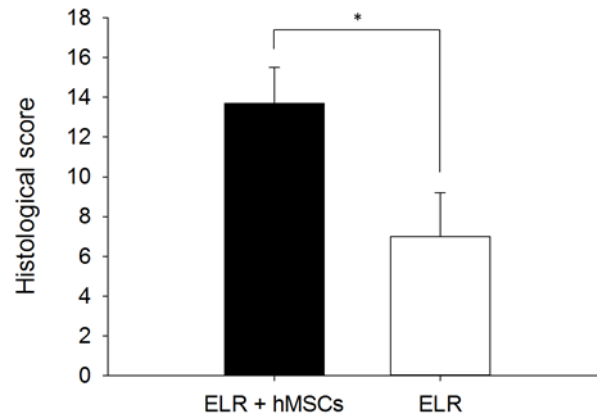
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2 **Figure 6.** Von Kossa staining of a sample from the experimental group showing
3 complete regeneration of articular cartilage (white dotted line) with areas of ossification
4 (yellow dotted line) beneath the cartilage. Scale bar corresponds to 250 μm .

5 3.4.2. Histological evaluation using the modified Wakitani grading scale

6 A statistical blind study of the histological sections using the modified Wakitani scale
7 gave a total score of 13.7 points for the defects from the experimental group (ELR +
8 hMSCs), which is close to the score for healthy cartilage (15 points). In contrast,
9 evaluation of the defects from the control group (ELR) gave a score of 7 points, with a
10 statistically significant difference ($p < 0.01$) in the overall score between experimental

1 and control groups (Figure 7). Complete scores from the histological evaluation are
2 shown in Table S-5 (Supplementary Material).

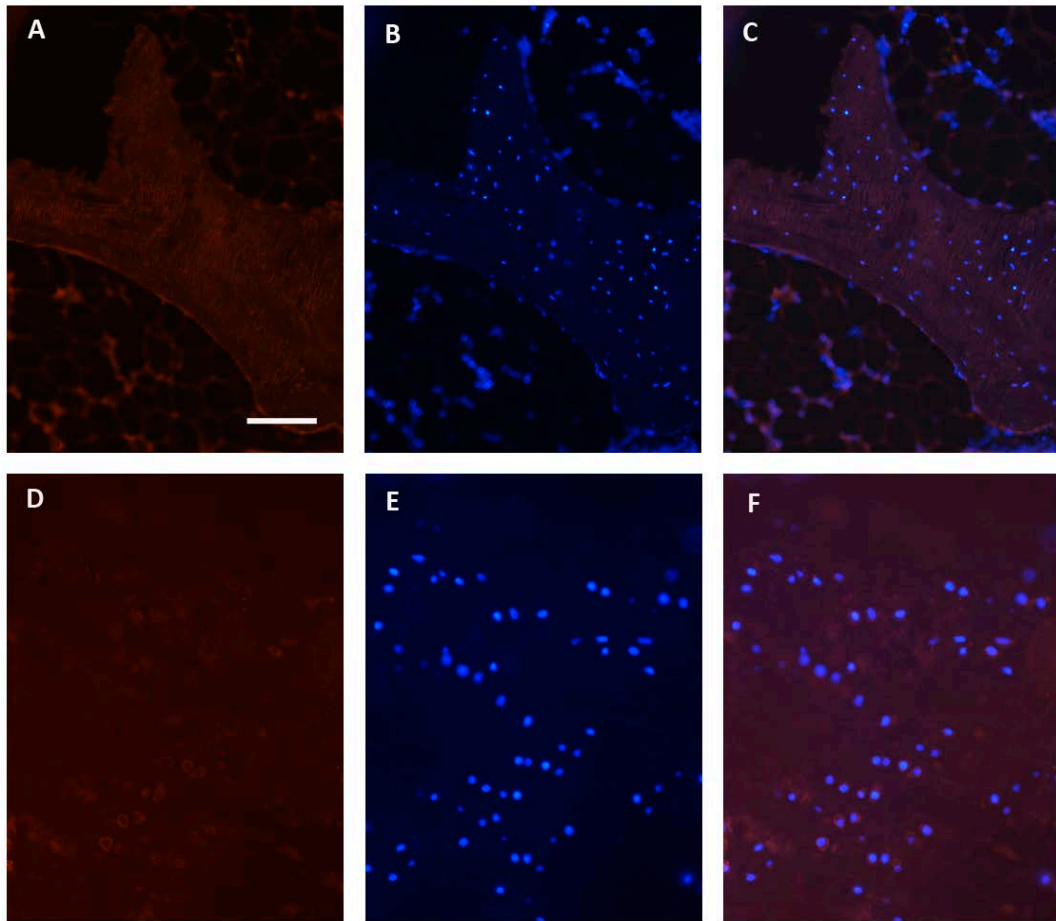


3

4 **Figure 7.** Comparison between the histological scores obtained for the experimental
5 (ELR + hMSCs) and control (ELR) groups, according to the modified Wakitani scale
6 (* $p < 0.01$).

7 3.4.3. Immunofluorescence analysis

8 The immunofluorescence results, obtained by combining antibodies versus the human
9 mitochondrial marker (red) and DAPI staining (blue), showed that hMSCs were
10 involved in regeneration of the osteochondral defect, since cells have a chondrocyte-like
11 morphology and arrangement, integrating the *de novo* formed tissue. This was only
12 observed in sections from the experimental group, with no fluorescence being detected
13 in the control group (Figure 8).



1

2 **Figure 8.** Immunofluorescence images to human mitochondrial marker, in red (A, D),
3 DAPI, in blue (B, E), an merged images (C, F) taken from animals of the control group
4 (ELR, A-C) and the experimental group (ELR + hMSC, D-E) showing the presence of
5 hMSCs in the regenerated tissue of animals from the experimental group. Scale bar =
6 125 μm for A-C, and 50 μm for D-F.

7 **DISCUSSION**

8 We have described the use of MSCs encapsulated within an ELR-based hydrogel to
9 regenerate an osteochondral defect for the first time. Although there are previous studies

1 regarding the treatment of this type of lesions with ELRs, none of them included any
2 type of cells inside the scaffold, and the outcomes observed were not very promising,
3 being similar to those for the control group.^{35,36} Furthermore, both studies involved the
4 use of chemically cross-linked hydrogels, which makes them harder to handle and may
5 lead to toxic sub-products. Moreover, in one case the ELR-based hydrogel was not
6 formed *in situ*, which compromises the adaptability to the shape of the defect, and the
7 use of genipin as cross-linker increases the expense considerably.³⁵ In our case the
8 network sustaining the hydrogel is formed via physical cross-linking (hydrophobic
9 interactions) above the Tt, e.g., at physiological temperature. As such, this system is
10 highly suitable for non-invasive techniques due to its injectability below the Tt. In
11 addition, biofunctionalization of the ELR by the genetic fusion of a 12-mer peptide
12 containing the RGD cell-adhesion sequence provides a cell-friendly environment for
13 hMSCs.

14 This approach represents a significant advance in the regeneration of osteochondral
15 injuries because it is widely thought that biomaterials should simulate the properties of
16 cartilage and subchondral bone, thus requiring biphasic³⁷ or even triphasic³⁸ scaffolds
17 that must comprise biomaterials with different features to recapitulate the osteochondral
18 interface and promote healing. However, here we show that the use of an injectable
19 ELR-based single hydrogel is an efficient approach in regenerative medicine as this
20 biomaterial serves only as a temporary microenvironment and does not need to have the
21 same properties as the native tissues.³⁹

1 Regeneration of the injured cartilage was successfully studied using a 1.5T MRI
2 instrument, without contrast agents, similarly to analogous models.^{40,41} This study
3 showed almost complete regeneration of the damaged tissue in the experimental group
4 (ELR + hMSCs) after three months. It was also possible to study the development of
5 subchondral bone with high precision by CT, thereby demonstrating regeneration of the
6 tissue and emphasizing the importance of this procedure for assessing regeneration of
7 osteochondral injuries in combination with MRI. This may allow more invasive
8 methods, such as biopsy, to be avoided.⁴²

9 With respect to the histological findings, the experimental (ELR + hMSCs) and control
10 (ELR) groups obtained 13.7 and 7 points, respectively, on the Wakitani scale, at three
11 months post-implantation. We chose this grading scale due to its simplicity in
12 comparison to other systems. Taking into account that the maximum score on this scale
13 is 15 points (Table S-2), we can calculate the percentage regeneration as the ratio
14 between the obtained and maximum values. Thus, we obtained a value of 91% for the
15 experimental group and 47% for the control group. This means that we can compare the
16 outcomes of osteochondral regeneration with MSCs (auto-, allo- or xenotransplanted),
17 at the same timepoint (3 months), even when different grading scales and/or animal
18 models are used, especially when considering that regeneration may vary between
19 species. For instance, Nakamura *et al.* created an osteochondral defect in both legs of
20 pigs, subsequently treating one knee with allogeneic MSCs while leaving the other as a
21 control. Their results, according to the same histological scoring scale as used in our
22 work, showed 57% regeneration for the experimental joint and 10% regeneration for the

1 control joint.³⁴ In another study, Koga *et al.* implanted allogeneic MSCs within a
2 collagen scaffold in rabbit knees, with the collagen hydrogel alone as control. Their
3 histological scoring (unmodified Wakitani scale) gave a regeneration of 89% and 21%
4 for the experimental and control groups, respectively.⁴³ Similarly, Mazaki *et al.*
5 described the use of allogeneic MSCs embedded in a gelatin hydrogel, obtaining 66%
6 regeneration in this case and 42% when using the gelatin hydrogel alone (modified
7 ICRS scale).⁴⁴

8 Other hydrogels have also been used in osteochondral regeneration. For instance, Miller
9 *et al.* studied the ability of an injectable peptide hydrogel combined with chondrogenic
10 factors and allogeneic MSCs to regenerate an osteochondral defect in rabbits, finding
11 poorer repair when MSCs were included in the hydrogel.⁴⁵ For their part, D'Este *et al.*
12 evaluated an injectable thermoresponsive hyaluronan/pNIPAAm hydrogel in an
13 analogous system, but not including MSCs. Their results showed better regeneration in
14 the non-treated (75%) than in the hydrogel-treated defects (70%).⁴⁶ Although these
15 authors claim that this biomaterial is biocompatible and does not interfere with the
16 intrinsic healing response, its implantation does not result in an improvement and thus it
17 is not very likely to be accepted by clinicians. In another study, Levingstone *et al.*
18 demonstrated the use of collagen-based scaffolds combined with other biomaterials
19 (multi-phasic scaffold) to recapitulate the osteochondral interface, without including
20 MSCs.⁴⁷ They reported 80% regeneration of defects treated with the cited scaffold, as
21 opposed to the 44% observed in the control non-treated defects. In contrast, Pulkkinen
22 *et al.* observed no significant differences in terms of histological assessment between

1 treatment of an osteochondral defect with recombinant human type II collagen
2 hydrogels embedding autologous chondrocytes and the spontaneously repaired tissue.⁴⁸
3 In the light of all these comparisons (see Table S-6, Supplementary Information), we
4 can conclude that our preliminary study represents a novel non-invasive therapy for the
5 treatment of injuries affecting articular cartilage with very promising results that are
6 better than those found in the literature in every case. One of the reasons that could
7 explain the differences between our study and others is the use of a bioactive ELR-
8 based hydrogel containing RGD cell-adhesion sequences, which therefore serves as an
9 ECM-like vehicle for hMSCs and provides a cell-friendly environment that supports
10 their regenerative potential. However, due to the subjectivity of histological scoring,
11 particular care must be taken when drawing conclusions from a direct comparison of the
12 results from different studies.

13 It is important to highlight the xenogeneic nature of the animal model used in this study.
14 This kind of cell therapy has been proposed as a replacement for allogeneic and
15 autologous bone and cartilage implantation, which have some limitations, especially a
16 shortage of donor tissue and the time-consuming nature of the procedure. Xenogeneic
17 MSCs have been used as undifferentiated cells, such as in our study, showing better
18 regeneration outcomes and higher survival than differentiated hMSCs.^{13,49} However,
19 Jang *et al.* showed good regeneration (75%) when including chondrocyte-differentiated
20 hMSCs in a biphasic composite (hydroxyapatite and platelet-rich fibrin glue) scaffold.⁵⁰
21 Similarly, the literature review by Li *et al.* concluded that porcine MSCs downregulate
22 T cell responses *in vitro*, which could also be reproduced *in vivo*, hence being a feasible

1 approach in clinical applications.¹² In contrast, Pei *et al.* reported a detrimental effect of
2 xenotransplanted MSCs in comparison to their non-treated control.¹⁴ In this case, we
3 believe that the influence of the matrix used as cell carrier was not considered as a
4 source of this effect and should be studied in more detail.

5 Moreover, the use of xenogeneic hMSCs allowed us to track the implanted cells in the
6 articular cartilage of the rabbit using a novel immunofluorescence technique that
7 involves the use of an antibody towards a specific human mitochondrial marker, thus
8 discerning between transplanted and host cells. This method allowed us to detect viable
9 and engrafted hMSCs after three months, thus proving that the ELR-based hydrogel can
10 be used as a successful cell carrier in which cells can differentiate and regenerate
11 damaged tissue. Our results therefore confirm that MSCs can overcome inter-species
12 barriers. In addition, we observed that the implanted human cells show a chondrocyte-
13 like shape and arrangement, similar to that of native cartilage, thus suggesting that
14 differentiation into a chondral cell lineage has taken place, which is in good agreement
15 with previous findings by Koga *et al.*⁴³

16 In summary, this work shows the potential effectiveness of a tissue-engineering
17 approach for regeneration of a critical-size osteochondral defect, in terms of
18 morphology and structure, upon combining hMSCs with a recombinantly developed
19 ELR-based cell-carrier hydrogel. The comparison with other approaches found in the
20 literature shows a more hyaline-like regeneration, and the closest previous result (89%,
21 by Koga *et al.*) involves the use of a non-injectable scaffold, which limits its application
22 in non-invasive therapies. This system provides a bioactive scaffold in which cells may

1 be embedded for accurate delivery to the injury site to promote tissue-engineered
2 regeneration of native-like hyaline cartilage. Further studies will be performed to
3 evaluate the functionality of regenerated articular cartilage through biomechanical tests,
4 and to determine the biochemical composition of the repaired tissue before translating
5 this system into larger animal models or humans.

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14 **CONFLICT OF INTERESTS**

15 The authors have no competing interests to declare.

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