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Histology and Histopathology

From Cell Biology to Tissue Engineering

Differentiation of human mesenchymal stromal cells cultured on collagen sponges for cartilage repair

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Summary. Aim: The aim of this study was to evaluate proliferation and chondrogenic differentiation of human bone-marrow mesenchymal stromal cells (hBMSCs) cultured on collagen biomaterials. Materials and Methods: hBMSCs were seeded on five different collagen (Col) sponges: C1C2 (types I and II Col), C1C2HS (types I and II Col plus heparan sulphate (HS)), C1C2CHS (types I and II Col plus chondroitin sulphate (CHS)), C1-OLH3 (type I Col plus low molecular weight heparin) and C1CHS (type I Col plus CHS). The resulting constructs were analyzed by histological and immunohistochemical staining, molecular biology and electron microscopy. Col released into culture media was measured by a dye-binding method. Results: hBMSCs on biomaterials C1C2, C1C2HS and C1C2CHS had more capacity to attach, proliferate and synthesize Col II and proteoglycans in the extracellular matrix (ECM) than on C1-OLH3 and C1CHS. The presence of aggrecan was detected only at the gene level. Total Col liberated by the cells in the supernatants in all scaffold cultures was detected. The level of Col I in the ECM was lower in C1-OLH3 and that of Col II was highest in C1C2 and C1C2HS. Electron microscopy showed differently shaped cells, from rounded to flattened, in all constructs. Col fibers in bundles were observed in C1C2CHS by transmission electron microscopy. Conclusions: The results show that Col I and Col II (C1C2, C1C2HS and C1C2CHS) biomaterials allowed cell proliferation and chondrogenic-like differentiation of hBMSCs at an early stage. Constructs cultured on C1C2HS and C1C2CHS showed better cartilage-like phenotype than the other ones.

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Abbreviations. 3D, three-dimensional; AB, alcian blue; Agg/AGG, aggrecan; BMSCs, bone-marrow mesenchymal stromal cells; CHS, chondroitin sulphate; Col, collagen; Col I/COLI, type I collagen; Col II/COLII, type II collagen; Col X/COLX, type X collagen; C1C2, biomaterials of types I and II collagen; C1C2HS, biomaterials of types I and II collagen plus heparan sulphate; C1C2CHS, biomaterials of types I and II collagen plus chondroitin sulphate; C1-OLH3, biomaterials of type I collagen plus low molecular weight heparin; C1CHS, biomaterials of type I collagen plus chondroitin sulphate; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, foetal bovine serum; GAGs, glycosaminoglycans; H-E, hematoxylin and eosin; hBMSCs, human bone-marrow mesenchymal stromal cells; HS, heparan sulphate; MT, Masson's trichrome; OA, osteoarthritis; PCNA, proliferating cell nuclear antigen; PGs, proteoglycans; q-PCR, real time quantitative PCR; REL, Relative Expression Levels; RT-PCR, reverse transcriptase PCR; SO, Safranin O; SEM, scanning electron microscopy; SOX9, SRY (sex determining region Y), box 9; TBP, TATA binding protein; TGFβ-3, Transforming growth factor β-3; TEM, transmission electron microscopy

Key words: Tissue Engineering, Osteoarthritis, Mesenchymal Stromal Cells, Biomaterials, Chondrogenesis

Introduction

Hyaline cartilage is composed of chondrocytes embedded in a self-produced extracellular matrix (ECM), primarily composed of proteoglycans (PGs) and a fibrillar collagen (Col) network in which type II Col represents approximately 90% of the Col mass (Grigolo et al., 2002a; Negri et al., 2007; Han et al., 2011).

Injured cartilage has serious limitations for self-repair (Zhang et al., 2012) due to its intrinsic characteristics (Fuss et al., 2000; Lee et al., 2006; Negri et al., 2007). Therefore, degradation of the ECM molecular components due to impairment of chondrocyte function or cell death can result in loss of tissue function and lead to osteoarthritis (OA) (Chen et al., 2001; Hollander et al., 2010).

OA is a chronic degenerative joint disorder characterized by pain and disability and has a worldwide impact (Matsumoto et al., 2009; Kock et al., 2012). Therapeutic and surgical treatments (Ehlers et al., 1999; Hunziker, 2002; Fuentes-Boquete et al., 2007; Fuentes-Boquete et al., 2008; Maher et al., 2010) have thus far led to variable and unsatisfactory outcomes (Tuli et al., 2003; Steinert et al., 2007; Maher et al., 2010; Cavallo et al., 2012; Kock et al., 2012).

Cartilage tissue engineering provides a promising avenue for restoring, replacing or regenerating defective cartilage using scaffolds, cells and cell factors to construct three-dimensional (3D) engineered tissues (Chan and Leong, 2008).

Col sponges have been reported to be suitable biomaterials for cartilage engineering (Hunziker, 2002; Claus et al., 2012; Xu et al., 2012). Col biomaterials have also been supplemented for improved cell viability (Chan and Leong, 2008), including a mixed biomaterial of Col and glycosaminoglycans (GAGs) (Jin et al., 2007).

There is controversy in the use of chondrocytes for cartilage tissue engineering, with bone-marrow mesenchymal stromal cells (BMSCs) being the preferred alternative (Varghese et al., 2008; Markway et al., 2010).

The immunogenic, anti-inflammatory and immunosuppressive characteristics (Hunziker, 2002; Hollander et al., 2010; Ohishi and Schipani, 2010) of human BMSCs (hBMSCs) and their capacity to differentiate into different mesodermal tissues, including cartilage (Wakitani et al., 2002; Hermida-Gomez et al., 2011; Cavallo et al., 2012; Cicione et al., 2015), make them ideal candidates. Transforming growth factor β-3 (TGFβ-3) has been widely utilized for chondrogenic differentiation (Lisignoli et al., 2005; Lee, et al., 2010a; Kock et al., 2012; Melrose et al., 2012).

The aim of this study was to evaluate the capacity for proliferation and chondrogenic differentiation of hBMSCs cultured on biomaterials of Col I and Col II mixed with GAG to form constructs useful for cartilage tissue engineering.

Materials and methods

Isolation and culture of mesenchymal stromal cells

Bone marrow samples used to isolate hBMSCs were obtained from 21 patients (mean age 74.66±10.25) undergoing total hip replacement due to OA. Samples were provided by the Orthopaedic Department of University Hospital Complex A Coruña. The local ethics committee approved this study and informed consent was obtained from all donors.

hBMSCs were extracted by washing the bone marrow with Dulbecco's modified Eagle's medium (DMEM; Lonza, Spain) and cultured in the same medium as described by Hermida-Gomez et al. (2011). When the hBMSCs became 80% confluent in culture, they were sub-cultured. To perform the studies, cells at 3rd passage were unequally pooled from different donors, thus avoiding interdonor variability.

Cell characterization

hBMSCs were characterized by flow cytometry (pool of 4 donors: mean age 76.25±2.87) and for multipotentiality (pool of 17 donors: mean age 74.29±11.36), as assessed by their capability to differentiate toward different cell types, as previously described (Díaz-Prado et al., 2010) (Figs. 1, 2). Third-passage hBMSCs were differentiated toward chondrocyte, adipocyte and osteoblast lineages, during 21 days using Commercial Chondrogenic, Adipogenic and Osteogenic Differentiation media (Lonza, Biowhittaker, Belgium). Differentiation was compared to cells cultured in DMEM with 20% foetal bovine serum (FBS) (Sigma–Aldrich, St. Louis, MO, USA). Adipogenic, osteogenic and chondrogenic differentiation were evaluated by histochemistry, immunohistochemistry and molecular biology.

Cell culture on biomaterials

After the hBMSCs were characterized, two pools of cells from 8 donors used in the multi-potential characterization were cultured on five different Col biomaterials (Opocrin S.p.A., Modena, Italy; Fig. 3A): C1C2 (types I and II Col), C1C2HS (types I and II Col plus heparan sulphate (HS)), C1C2CHS (types I and II Col plus chondroitin sulphate (CHS)), C1-OLH3 (type I Col plus low molecular weight heparin) and C1CHS (type I Col plus CHS). hBMSCs were cultured on the surface of 1x1 cm2 sponges (200,000 cells/cm^2; Fig. 3B) for 30 days under normoxia conditions (humidified atmosphere with 5% CO_2). Chondrogenesis was assessed by culturing hBMSCs in the presence of commercial hMSC chondrogenic induction medium with 10 ng/ml of human TGFβ-3 (Prospec-Tany Technogene

Ltd., Rehovot, Israel). For negative controls, free-cell biomaterials were cultured in the same growth medium and for the same period of time. Culture media were changed 2-3 times a week. The culture supernatants from each change of culture medium were assayed to detect extracellular secretion of Col using a dye-binding method described below. After 30 days, 6 independent replicates of each biomaterial were collected for histological and immunohistochemical assays, real time quantitative PCR (q-PCR) analyses and electron microscope studies, as described below.

Chondrogenic differentiation on biomaterials

Histological analysis

Constructs with cells and free-cell biomaterials were fixed in 4% formaldehyde and embedded in paraffin. To evaluate chondrogenesis, 4 μm-thick paraffin sections of biomaterials were deparaffinized in xylol, rehydrated in a graded series of ethanol and stained with hematoxylin and eosin (H-E), Masson's trichrome (MT), alcian blue (AB), Safranin O (SO) and Von Kossa (VK).

Immunohistochemical analysis

Paraffin sections (4 μm-thick) were incubated with primary antibodies to detect the presence of Col I (monoclonal clone), II (monoclonal clone), and type X Col (Col X) (monoclonal clone), aggrecan (Agg C-20) (monoclonal clone), matrix metalloproteinase 13 (MMP-13) (monoclonal clone) (all from Thermo Fisher Scientific, Madrid, Spain) and proliferating cell nuclear antigen (PCNA) (monoclonal clone) (Calbiochem, Madrid, Spain). The peroxidase/DAB ChemMate™ DAKO EnVision™ detection kit (Dako, Barcelona, Spain) was used to determine antigen-antibody interactions.

Histological and immunohistochemical evaluation of constructs

Samples were assessed by 3 independent observers using an optical microscope and the analiSIS® software (version D; Olympus, Germany). For study groups using H-E and MT staining, the percentage of cells in the total area of the biomaterial studied, the location (superficial or internal) of the cells in the biomaterial, and cell morphology and viability (by PCNA staining) were determined. The percentage of cells in the area of biomaterial studied was grouped by score: <1% (scored as 0), 1-24% (scored as 1), 25-49% (scored as 2), 50-74% (scored as 3) and 75-100% (scored as 4) (Sanjurjo-Rodríguez et al., 2014).

Histological and immunohistochemical results were expressed as semi-quantitative scores according to staining intensity: negative or absent (– or 0), weak (1+), moderate (2+) and strong (3+) (Sanjurjo-Rodríguez et al., 2014). To perform a global evaluation, staining and immunostaining values were calculated as the product of the categorized staining scores and percentage of cells; the values obtained were between 1 and 12: 1-4 lowest, 5-8 intermediate and 9-12 highest values (Table1).

RNA extraction, cDNA synthesis and quantitative Real Time PCR (q-PCR) analysis

Three constructs of each biomaterial were washed and sliced. The slices were placed in tubes with zirconium oxide grinding balls (Retsch, Haan, Germany) and frozen in liquid nitrogen. The tubes were then placed in a Mixer Mill MM200 (Retsch) to disintegrate the constructs. Total RNA was isolated from the homogenate using Trizol Reagent, following the manufacturer's protocol (Invitrogen, Barcelona, Spain). RNA was assessed for quantity at 260 nm using a NanoDrop™ spectrophotometer (Thermo Scientific, Madrid, Spain). The A260/A280 ratio was calculated to assess quality and purity. Total RNA was processed by reverse transcriptase PCR (RT-PCR).

Before RT-PCR, total RNA was treated with DNAse (Fermentas, Madrid, Spain) for complete removal of DNA contamination. The RT-PCR reaction was performed from total RNA following the manufacturer's instructions, using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen).

q-PCR analyses were performed on a LightCycler® 480 Instrument (Roche, Mannheim, Germany) using LightCycler 480 SYBR Green I Master (Roche), following the manufacturer's instructions. The primers

Table 1. Summary of histological and immunohistochemical results.

	% cells	% PCNA	Staining intensity or metachromasia							
			AB	SO	VK	Col I	Col II	Col X	MMP13	Agg C20
C1C2	3	9	6	9	0	6	9	9	0	0
C1C2HS	4	12	4	12	4	12	12	4	0	0
C1C2CHS	4	4	8	8	0	4	12	4	4	0
C1-OLH3	3	0	0	6	0	0	3	0	3	0
C1CHS	0	0	0	0	0	0	0	0	0	0

Staining: AB, alcian blue; SO, safranin O; VK, Von Kossa. Immunostaining: PCNA, proliferating cell nuclear antigen; Col I, type I collagen; Col II, type II collagen; Col X, type X collagen; MMP13, matrix metalloproteinase 13; Agg C20, aggrecan.

were purchased from Roche. TATA binding protein (TBP) (Roche) was used as the internal control housekeeping gene to normalize the amount of target cDNA. Primers of SRY (sex determining region Y), box 9 (SOX9), Agg (AGG) and Col II alpha 1 (COLII) genes, were used to evaluate chondrogenesis. To detect differentiation towards fibroblasts, Col I (COLI) primers were used. To detect hypertrophy, type X Col (COLX) was tested.

Data analysis was performed in triplicate using LightCycler 480 Relative Quantification software (Roche). Relative Expression Levels (REL) were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Electron microscope analysis

Samples for electron microscopy were processed in the *Servizo de Apoio á Investigación* at the University of A Coruña (SAI-UDC).

Scanning electron microscopy

Constructs with cells and free-cell biomaterials were fixed in 3% glutaraldehyde in cacodylate buffer. Before dehydration in a graded series of ethanol, the samples were fixed in osmium tetroxide. Samples were critical point dried by flooding with liquid CO_2 in a Bal-Tec CPD 030 (Balzers, Liechtenstein, Germany) and gold-sputtered with a coater Bal-Tec SCD 004 (Balzers). Samples were observed using a scanning electron microscope (SEM) JSM 6400 (JEOL, Tokyo, Japan).

Transmission electron microscopy

Constructs with cells and free-cell biomaterials were fixed in 3% glutaraldehyde in cacodylate buffer. Biomaterials were dehydrated in a graded series of acetone after fixation in osmium tetroxide. Samples were embedded in Spurr (Electron Microscopy Sciences, Hatfield, USA) and 60 nm thick Spurr sections were observed by TEM Jeol JEM 1010 (JEOL).

Collagen assay

The concentration of total Col secreted by the cells was determined in the supernatants at each change of culture medium, using a dye-binding method (Sircol™ Collagen Assay; Biocolor, City, UK) for analysis of acid and pepsin-soluble collagens, following the manufacturer's protocol. Supernatants from biomaterials cultured without cells were utilized as negative controls to detect any Col originating from biomaterial degradation.

Statistical analysis

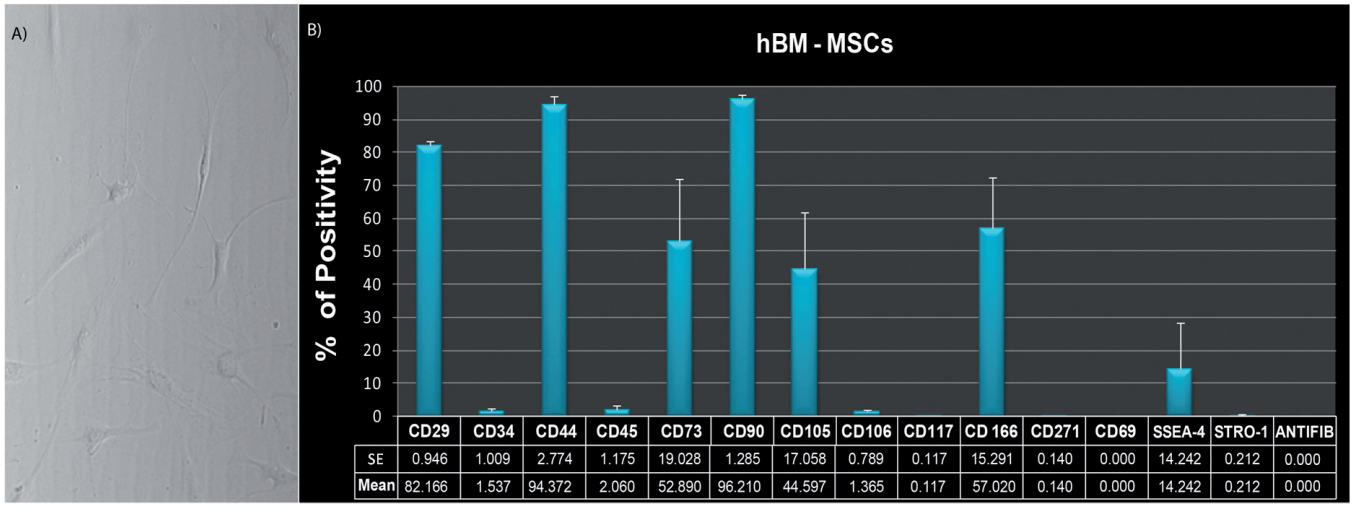
All statistical analyses were performed with SPSS 18.0 software for Windows, using non-parametric tests. p-values <0.05 were considered to be statistically significant. Results were expressed as the mean ± standard error (mean ± SE).

Results

Evaluation of the extracellular matrix

Proteoglycan detection

C1C2 and C1C2HS constructs showed the highest metachromasia with SO staining, indicating the presence of sulphated GAGs (Fig. 4, Table 1). C1C2CHS and C1-OLH3 constructs were highly stained, but those of



	CD29	CD34	CD44	CD45	CD73	CD90	CD105	CD106	CD117	CD 166	CD271	CD69	SSEA-4	STRO-1	ANTIFIB
SE	0.946	1.009	2.774	1.175	19.028	1.285	17.058	0.789	0.117	15.291	0.140	0.000	14.242	0.212	0.000
Mean	82.166	1.537	94.372	2.060	52.890	96.210	44.597	1.365	0.117	57.020	0.140	0.000	14.242	0.212	0.000

Fig. 1. A. Image of human bone-marrow mesenchymal stromal cells (ChBMSCs) in culture with their characteristic fibroblastic morphology. **B.** Percentage of positivity (mean and standard error (SE)) obtained by flow cytometry for markers characteristic of mesenchymal cells (CD29, CD44, CD73, CD90, CD105, CD106, CD117, CD166, CD271, CD69), embryonic cells (SSEA-4, STRO-1), hematopoietic cells (CD34, CD45) and antifibroblast. A, x 200

C1CHS were not (Fig. 4, Table 1). Biomaterial fibers were orthochromatically stained (Fig. 4, Table 1). AB staining showed the bigger area of staining in the ECM of cells grown on C1C2 and C1C2CHS (Fig. 4, Table 1), indicating the presence of high amounts of PGs. As seen in Fig. 4, the biomaterial fibers stained with higher intensity than the ECM. C1C2HS showed less staining intensity and C1CHS and C1-OLH3 did not stain (Fig. 4, Table 1). Agg immunostaining was negative in all constructs (Fig. 4, Table 1).

Free-cell controls were negative for AB, SO and Agg (Fig. 5).

Collagen assays

After measuring the total amount of Col liberated to supernatants from biomaterials cultured without cells, secreted-total Col (μg) liberated by cells was detected in all constructs (Fig. 6A). In C1C2, C1C2HS and C1-OLH3 constructs, cells progressively liberated Col up to a maximum and then decreased progressively. On day 11, C1C2, C1C2HS and C1-OLH3 had peaks of 13.99±4.77 μg, 11.10±7.50 μg and 38.10±6.13 μg, respectively (Fig. 6A). C1C2CHS was seen to have two peaks, one on day 4 (17.16±12.87 μg) and, following a few days of lower concentration, another one at day 21 (13.90±3.93 μg). C1CHS also had two peaks at day 4 (72.22±11.47 μg) and day 14 (20.50±3.42 μg) (Fig. 6A). Significant differences in total released collagen between biomaterials existed at days 4 (p=0.002), 11 (p=0.002), 14 (p=0.046) and 21 (p=0.007). Showing the highest Col concentration were the following: at day 4 and day 14, C1CHS (72.22±11.47 μg and 20.50±3.42 μg, respectively); at day 11, C1-OLH3 (38.100±6.130 μg); and at day 21, C1C2CHS (13.904±3.936 μg) (Fig. 6A).

Immunohistochemistry revealed that ECMs of C1C2

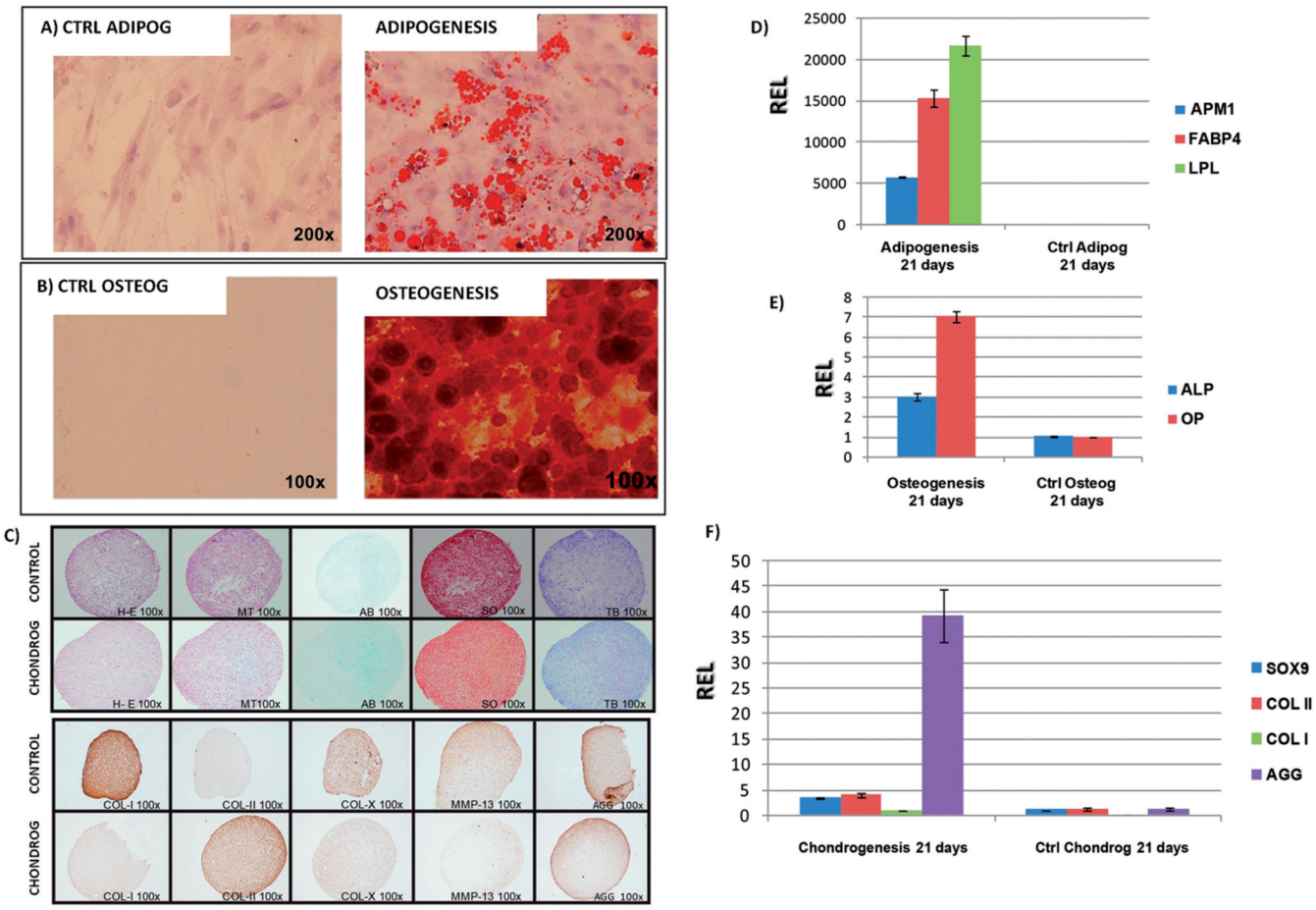


Fig. 2. Multipotential characterization. **A.** Images of adipogenic differentiation and control cultures (Ctrl Adipog) stained with oil red O. **B.** Images of osteogenic and control cultures (Ctrl Osteog) stained with alizarin red. **C.** Images of histological and immunohistochemistry stainings from chondrogenic differentiation (Chondrog) and control cultures (Control). **D.** REL of adipogenic characteristic genes in adipogenic differentiation and control cultures. **E.** REL of osteogenic characteristic genes in osteogenic differentiation and control cultures. **F.** REL of characteristic (COLII, SOX9, AGG) and non-characteristic (COLI) chondrocytic genes, in chondrogenic differentiation and control cultures (Ctrl Chondrog).

and C1C2HS constructs were intensely stained and cells of C1C2CHS had intracytoplasmic staining for Col I (Fig. 6B, Table 1). Cells grown on C1-OLH3 and C1CHS constructs were negative for Col I with immunostaining (Fig. 6B, Table 1). The ECMs of all constructs, except C1CHS, were positive for Col II immunostaining; C1C2, C1C2HS and C1C2CHS constructs being the most intensely stained (Fig. 6B, Table 1) and C1-OLH3 the least. Both cells and the ECM of C1C2 constructs were stained intensely for Col X while ECMs in C1C2HS and C1C2CHS stained weakly (Fig. 6B, Table 1). Cells grown on C1-OLH3 and C1CHS were negative for Col X immunostaining (Fig. 6, Table 1). Free-cell controls were weakly positive for Col I and Col II, except C1C2 that showed more positivity for Col II (Fig. 5). For Col X, all free-cell biomaterials presented positivity.

Evaluation of cellular element

Cell morphology assays

H-E and MT staining showed that the percentage of cells in the area of analysed biomaterial was higher than 50% in C1C2 and C1-OLH3 and higher than 75% in C1C2HS and C1C2CHS biomaterials, while that of C1CHS was below 1% (Fig. 7, Table 1). hBMSCs were able to grow on the surface and inside all biomaterials except C1CHS, where they grew only on the surface. In C1C2, C1C2HS, C1C2CHS and C1-OLH3 biomaterials, cell aggregates with surrounding ECM and consequent biomaterial degradation were found, except in C1-OLH3 (Fig. 7). With MT staining, large amounts of Col were found in the ECM, secreted by cells grown on C1C2, C1C2HS, C1C2CHS, but lesser amounts of Col with C1-

A

Biomaterials	Composition					Thickness
	Type I Col	Type II Col	HS	CHS	Heparin	
C1C2	X	X				1 mm
C1C2HS	X	X	X			
C1C2CHS	X	X		X		
C1-OLH3	X				X	
C1CHS	X			X		

B

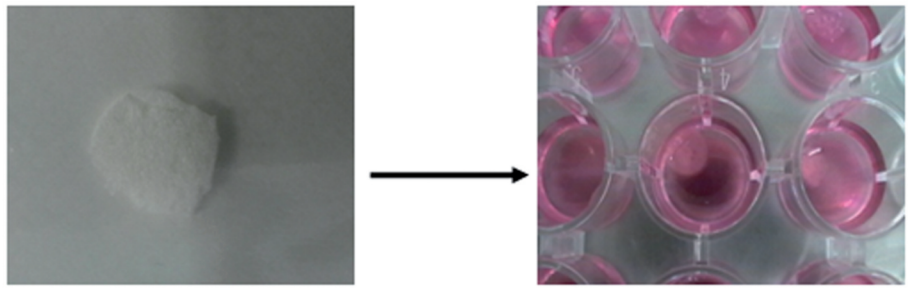


Fig. 3. A. Summary table of biomaterial composition and characteristics. **B.** Images of sponges before and after culture.

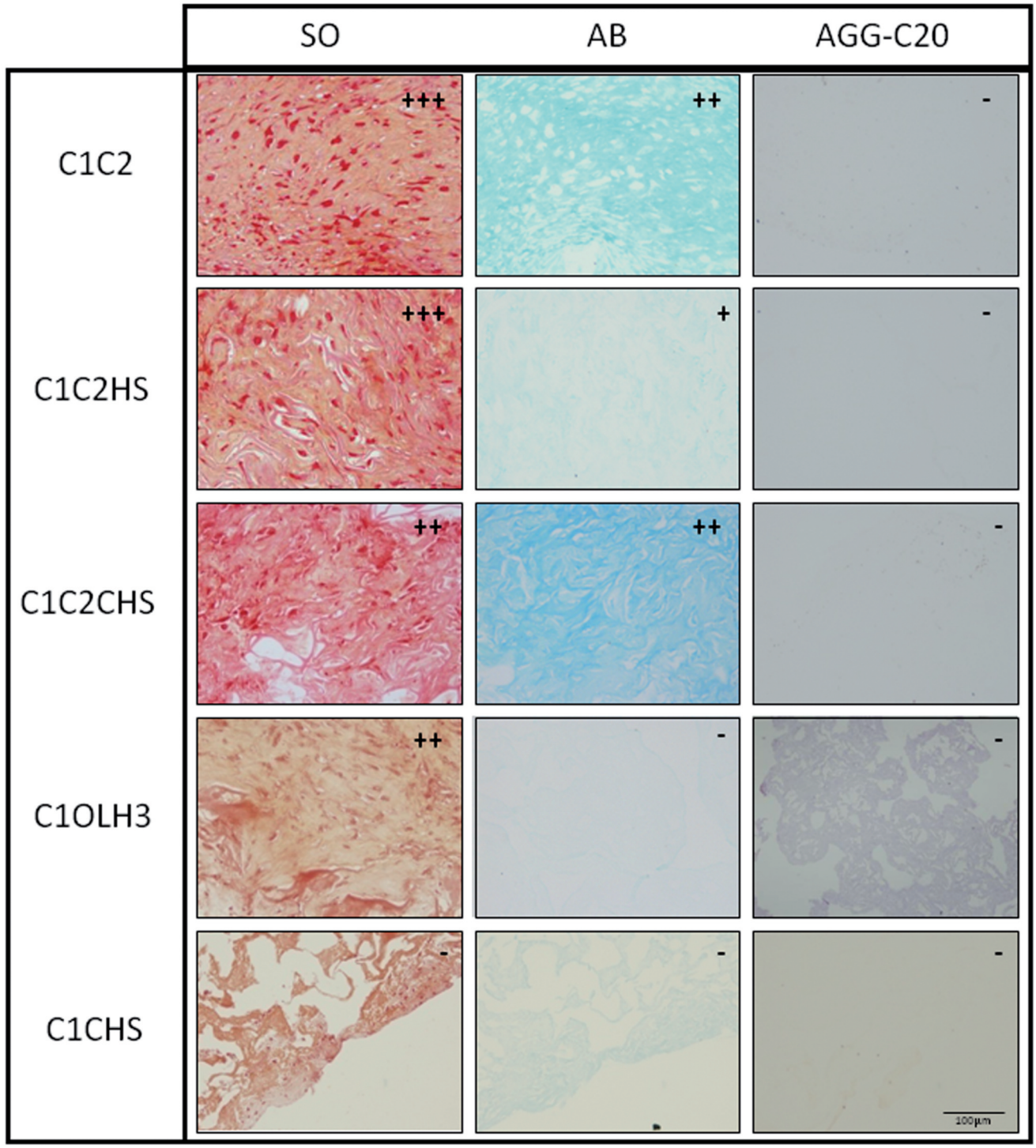


Fig. 4. Images of proteoglycans (PGs) stained by safranin O (SO; left column) and alcian blue (AB; middle column) histological stains, and aggrecan (Agg-C20; right column) immunostaining. From top to bottom the different types of scaffolds are shown. Semi-quantitative scores according to staining intensity or metachromasy: negative or absent (– or 0), weak (1+), moderate (2+) and strong (3+). Scale bar: 100 μm.

OLH3 (Fig. 7). The MT staining of C1CHS showed only biomaterial positivity. The fibers of all the biomaterials were stained more intensely than the ECM (Fig. 7).

Cell proliferation analyses

PCNA immunostaining detected many proliferative cells in all biomaterials, except C1CHS (Fig. 7, Table 1). C1C2 and C1C2HS had the most intense staining (Fig. 7, Table 1).

Detection of hypertrophy markers

Cells grown on the constructs were not positive for MMP-13 immunostaining, except for C1C2CHS and C1-OLH3, which showed weak intracytoplasmic positivity (Fig. 8, Table 1). Cells grown on all biomaterials were negative for VK staining except C1C2HS, which showed slight positivity, indicating no mineralization in all constructs (Fig. 8, Table 1). Col X was negative in C1-OLH3, C1CHS and in cells of

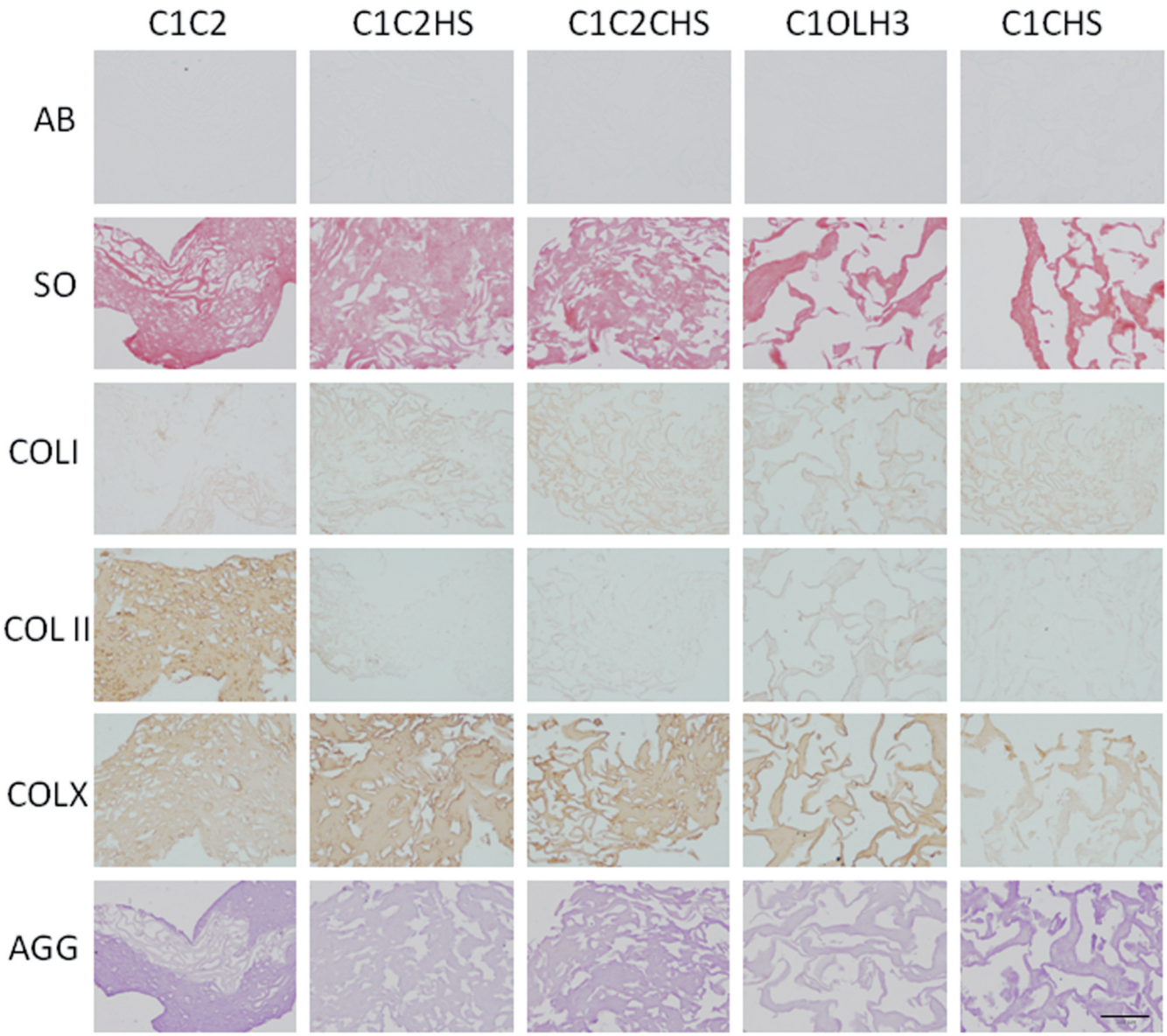


Fig. 5. Images of free-cell scaffolds stainings and immunostainings: AB, alcian blue; SO, safranin O; Col I, type I collagen; Col II, type II collagen; Col X, type X collagen; Agg C20, aggrecan. Scale bar: 100 μm

C1C2HS and C1C2CHS. On the other hand, C1C2 was intensely stained for Col X (Fig. 6B, Table 1).

Gene expression studies

Significant differences were found between the biomaterials for expression of the typical chondrogenic genes, SOX9 (p=0.039) and COLII (p=0.043). Cells grown on C1CHS biomaterial expressed the lowest REL for SOX9 (0.089±0.001) and the highest (5.210±1.050) for C1C2HS (Fig. 9A). Cells grown on C1CHS also expressed the lowest REL for COLII (0.155±0.007), while cells grown on C1C2 (6.130±0.000) and C1C2HS (5.670±0.000) showed the highest REL for COLll (Fig. 9A). The highest REL for the AGG gene was found in cells grown on C1C2HS (5.974±1.004) and the lowest was seen on C1-OLH3 (0.000±0.000) and C1CHS (0.000±0.000), but there were no significant differences between biomaterials (p=0.075) (Fig. 9A). However, there were significant differences between biomaterials in the REL of the COLI gene (p=0.021). Highest expression of COLI was found in cells for C1C2HS (0.805±0.127) and lowest in cells for C1-OLH3 (0.000±0.000) (Fig. 9A). Significant differences were found for COLX (p=0.008), REL were higher for C1C2 (162.488±12.365) than for C1C2HS (2.043±0.071) and C1C2CHS (1.151±0.150). COLX expression was not found for C1-OLH3 and C1CHS.

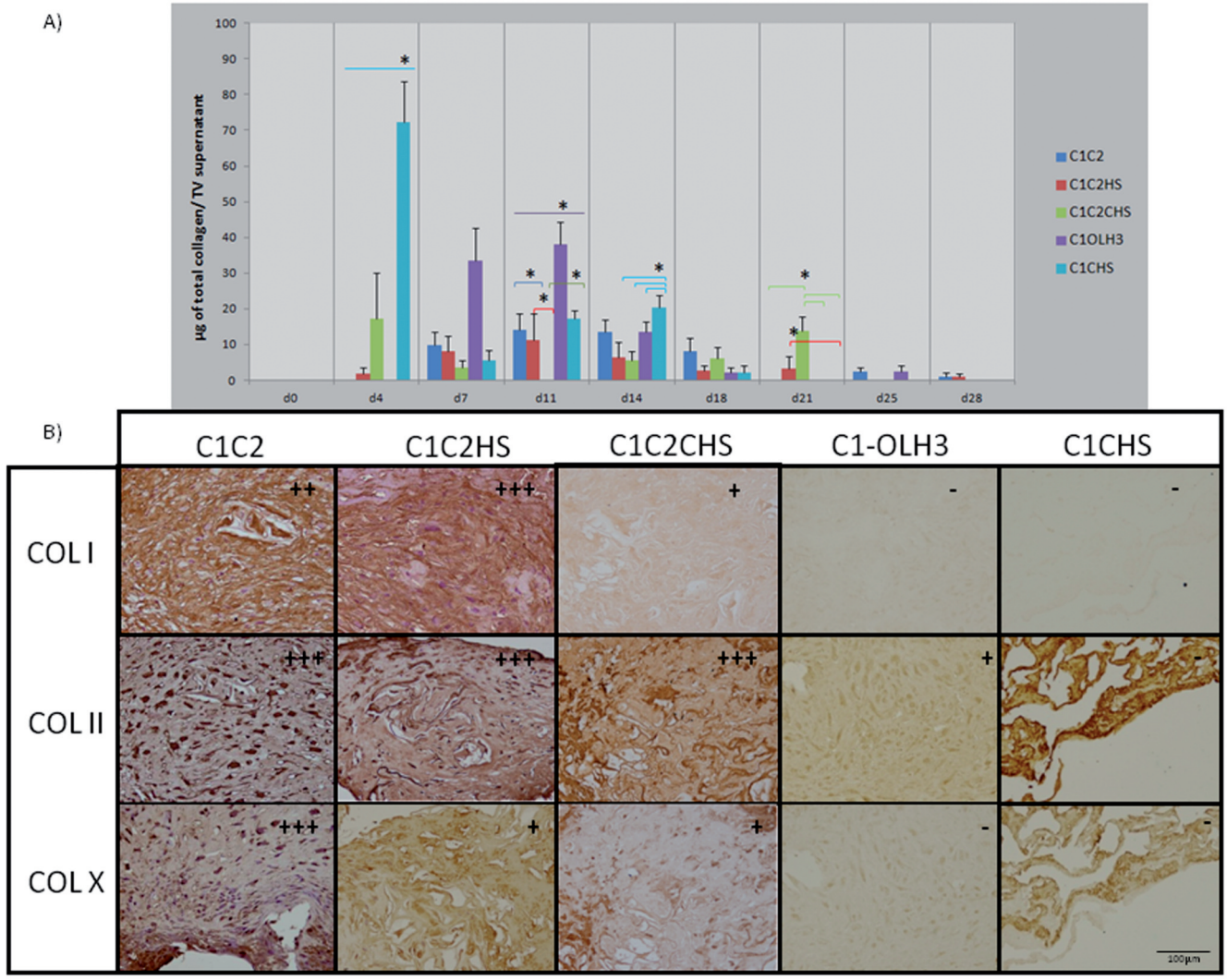


Fig. 6. A. Mean of total collagen (µg) content measured in the total culture media supernatant volume (TV), over days in culture of the different biomaterial constructs. * Statistical differences. **B.** Images of Col I (top row), Col II (middle row) and Col X (bottom row) immunohistochemical staining in all the scaffolds (from left to right: the different types of scaffolds). Semi-quantitative scores according to immunostaining intensity: negative or absent (– or 0), weak (1+), moderate (2+) and strong (3+). Scale bar: 100 µm.

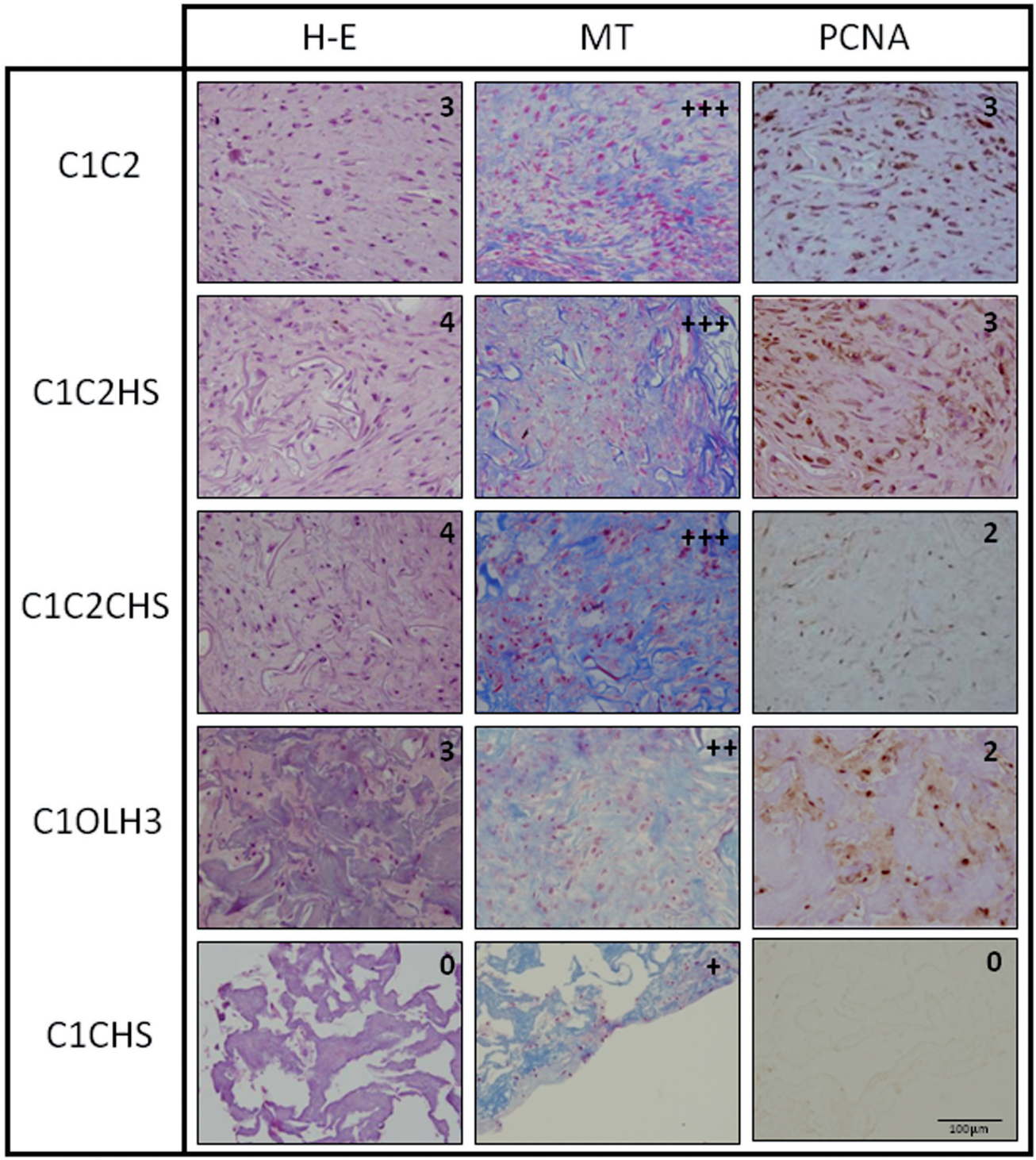


Fig. 7. Images obtained from the different constructs by histological and immunohistochemical staining. From top to bottom the different types of scaffolds are shown. From left to right, hematoxylin and eosin (H-E; left column) and Masson's trichrome (MT, middle column) histological stainings and proliferating cell nuclear antigen (PCNA; right column) immunostaining are shown. Semi-quantitative scores according to staining intensity: negative or absent (– or 0), weak (1+), moderate (2+) and strong (3+). Percentage of cells and PCNA immunostaing: <1% (0), 1-24% (1), 25-49% (2), 50-74% (3) and 75-100% (scored as 4). Scale bar: 100 μm.

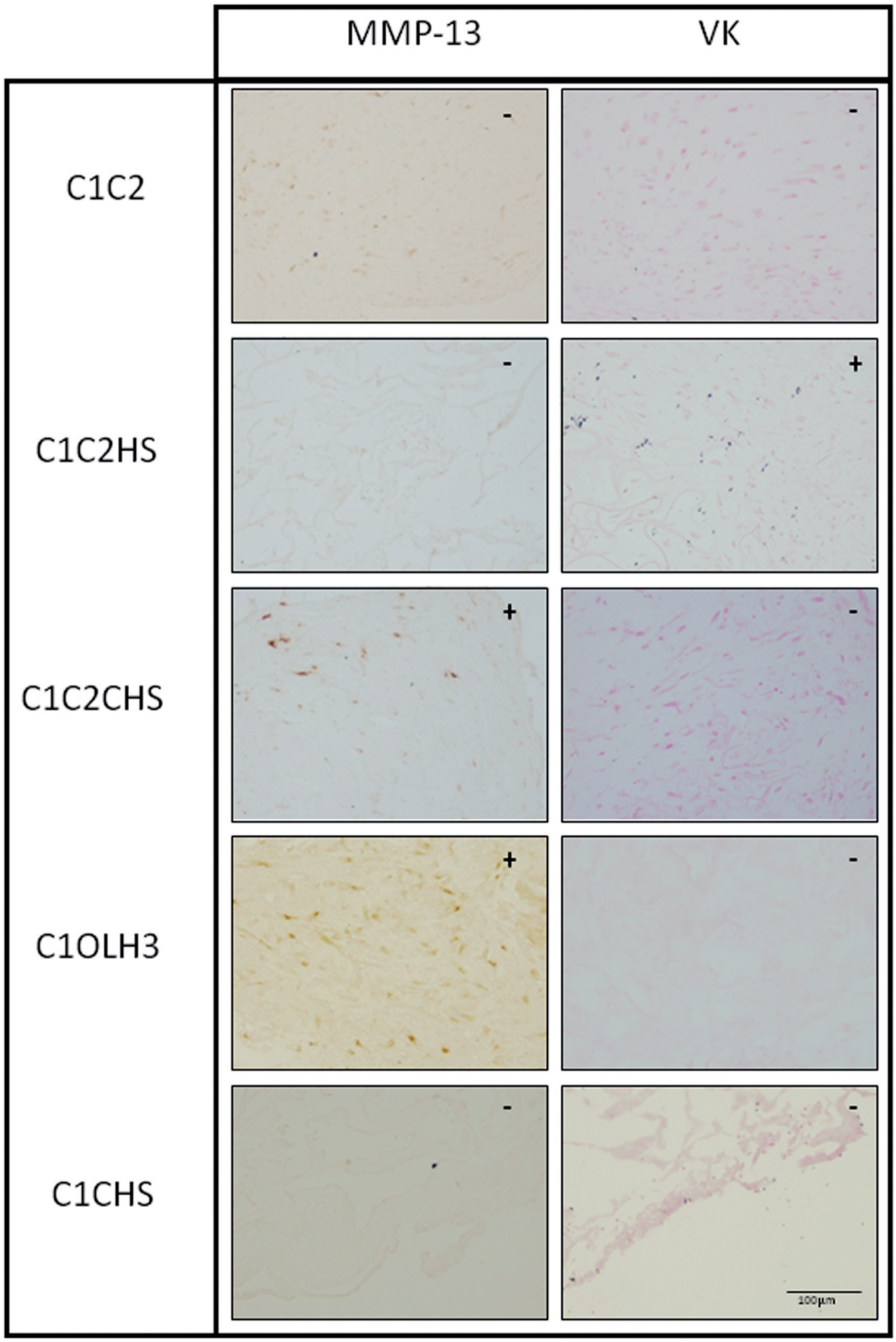


Fig. 8. Images obtained from the different constructs by matrix metalloproteinase 13 immunostaining (MMP-13, left column) and Von Kossa staining (VK, right column). Semi-quantitative scores according to immunostaining intensity: negative or absent (– or 0), weak (1+), moderate (2+) and strong (3+). Scale bar: 100 μm.

Morphometric and ultrastructural evaluations

Morphometric analysis performed using SEM on the constructs showed that cells had variable morphologies in all the biomaterials.

Accumulations of roughly fused spherical material appeared in the extracellular space in all the constructs (Fig. 10) and small spheres that did not appear in the respective controls often appeared near the cells (Fig. 10A,C,E,H-J,L-O). These accumulations were electron-dense because of their high osmium tetroxide affinity, indicating high lipid content. The electron-dense accumulations are also present in TEM. This may indicate that the vesicles of extracellular material were actively secreted by cells.

In C1C2 cells were flattened with short prolongations (Fig. 10B). C1C2CHS also presented flattened cells but with large prolongations from the cellular wall (Fig. 10G,H). In the other biomaterials cells were rounded and only in C1-OLH3 did we find large prolongations (Fig. 10K).

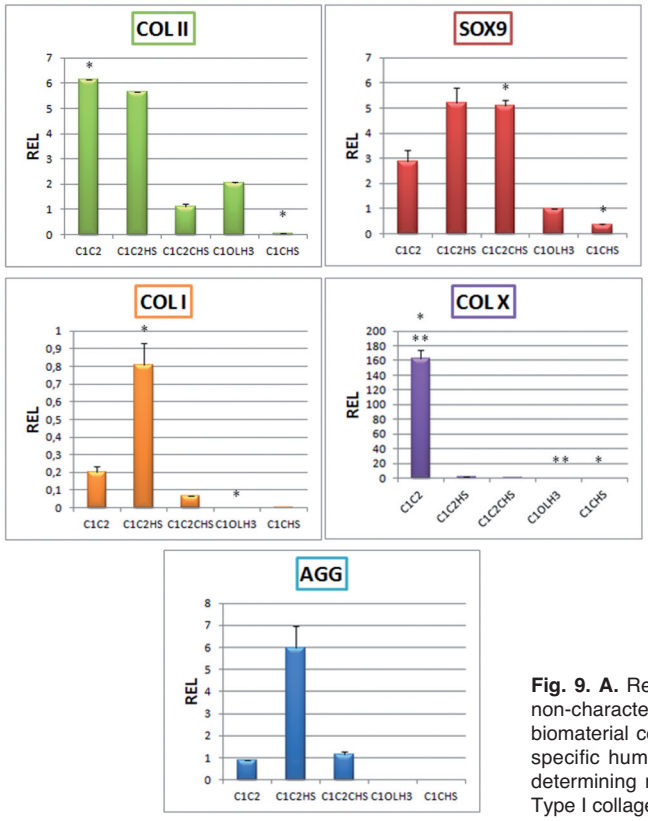
Ultrastructural analysis of the constructs using TEM showed cells throughout all biomaterials and that some parts of all biomaterials were degraded. Cells were proliferative, as seen in Fig. 11, and were oval shaped. Cells showed abundant intracellular organelles like mitochondria (Fig. 11A,D,E,I-M), indicating high cell activity. Few small prolongations were seen around cells (Fig. 11A,D,E,K-M), and inside cells some electron-dense granules were found (Fig. 11A,B,D,E, G,H,K,Q). Inside the biomaterials, highly vacuolated apoptotic cells (Fig. 11;AP) and cellular debris containing many mitochondria and electron-dense granules (Fig. 11B,F,P) were found.

Inside the C1C2 construct, cells were found between biomaterial fibers (Fig. 11A,B,C;Sc) and ECM was not detected. Oval-shaped cells in the C1C2HS construct were surrounded by biomaterial (Fig. 11D-F;Sc) and ECM was not detected.

Similar to C1C2, cells in C1C2HS contained abundant secretions (Fig. 11D), intracytoplasmic electron-dense granules (Fig. 11D-F) and a few small prolongations (Fig. 11D,E). In these cells, mitochondria were fewer and vacuoles more patent (Fig. 11D,E). Cellular debris was also present (Fig. 11F).

Inside C1C2CHS, cells were found closely attached to the biomaterial fibers (Fig. 11G,H;Sc); these cells showed an irregular shape and much ECM. In this ECM we observed Col fibers in bundles (Fig. 11G) running in various planes. The cells contained many electron-dense granules (Fig. 11G,H) and organelles (Fig. 11I,J), particularly mitochondria, and an oval/rounded cellular

A



B

Primer	Sequence F (forward)	Sequence R (reverse)	% G/C F	% G/C R	Amplicon (bp)
TBP	5´gcccatagtgatctttgcagt3´	5´cgctggaactcgtctcacta3´	48%	55%	142 nt
COLII	5´gtgtcagggccaggatgt3´	5´tcccagtgtcacagacacagat3´	61%	50%	116 nt
SOX 9	5´gtacccgcacttgcacaac3´	5´tcgctctcgttcagaagtctc3´	58%	52%	72 nt
AGG	5' gcctacgaagcaggctatga3'	5' gcacgccataggtcctga3'	55%	61%	136 nt
COLI	5' gtgatgctggtcctgttggt3'	5' caccatcgtgagccttctct3'	55%	55%	123 nt
COLX	5' caccttctgcactgctcatc3'	5' ggcagcatattctcagatgga3'	55%	48%	104 nt

Fig. 9. A. Relative Expression Levels (REL) of characteristic (COLII, SOX9, AGG) and non-characteristic (COLI) chondrogenic genes and hypertrophic gene (COLX) in each biomaterial construct. **B.** Primer sequences and conditions of qPCR for amplification of specific human genes. TBP: Housekeeping, TATA binding protein; SOX9: SRY (sex determining region Y)-box 9 (SOX9); COLII: Type II collagen; AGG: Aggrecan; COLI: Type I collagen; COLX: Type X collagen. * or ** Statistical differences.

nucleus (Fig. 11;Nuc) with a prominent nucleolus. There were no apparent prolongations seen around the cells and only a little extracellular material (Fig. 11G).

The C1-OLH3 constructs had cells with different morphologies, from elongated to round (Fig. 11K-M) and no ECM. These cells were seen with no biomaterial surrounding them; proliferating cells were also seen (Fig. 11K). In the cytoplasm there were many vesicles containing material (Fig. 11L,M) and electron-dense granules (Fig. 11K). These cells possessed many prolongations (Fig. 11K-M) and no extruded material was seen. Inside the C1CHS construct we found apoptotic cells (Fig. 11P;AP) and long fibroblast-like cells (Fig. 11N,Q), closely attached to the biomaterial fibers (Fig. 11;Sc). These cells contained large numbers of electron-dense granules in the cytoplasm (Fig. 11Q) and no extruded material or ECM was visible.

Discussion

Since its emergence, tissue engineering has evolved as a multidisciplinary field with an overall aim of developing biological substitutes to restore, replace or regenerate defective tissues (Chan and Leong, 2008). There is great promise for the generation of functional cartilage substitutes using engineered tissue constructs *in vitro* for subsequent implantation *in vivo*. Nonetheless, constructs currently obtained by tissue engineering have key challenges remaining to be addressed (Tuli et al., 2003).

Stem cells, when used in combination with proper scaffolds and appropriate growth factors, could have a great impact on cartilage repair (Chen et al., 2011). According to the international guidelines (Dominici et al., 2006) BMSCs should be all (>95%) positive for

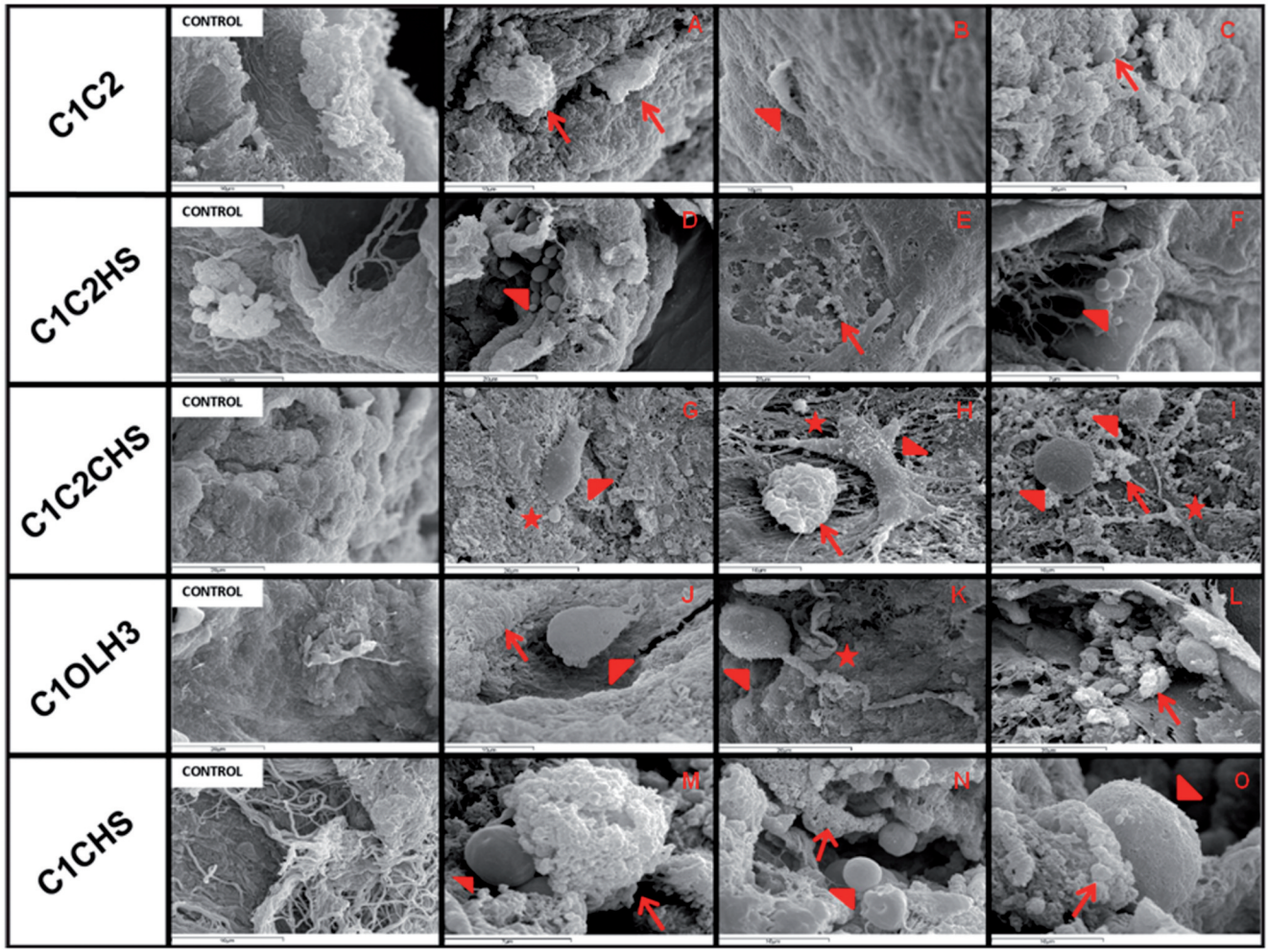


Fig. 10. Images obtained from the different constructs with cells and free-cell scaffolds (control) by scanning electron microscopy (SEM). From top to bottom the different types of scaffolds are shown. Arrowhead: cells; star: prolongations from the cellular wall; arrow: spheres of extracellular material. Scale bars: C1C2, C1C2HS and C1CHS controls and A, B, H, I, J, N, O, 10 μm; C1C2CHS and C1-OLH3 controls and C, D, E, G, K, 20 μm; F and M, 7 μm; L, 30 μm.

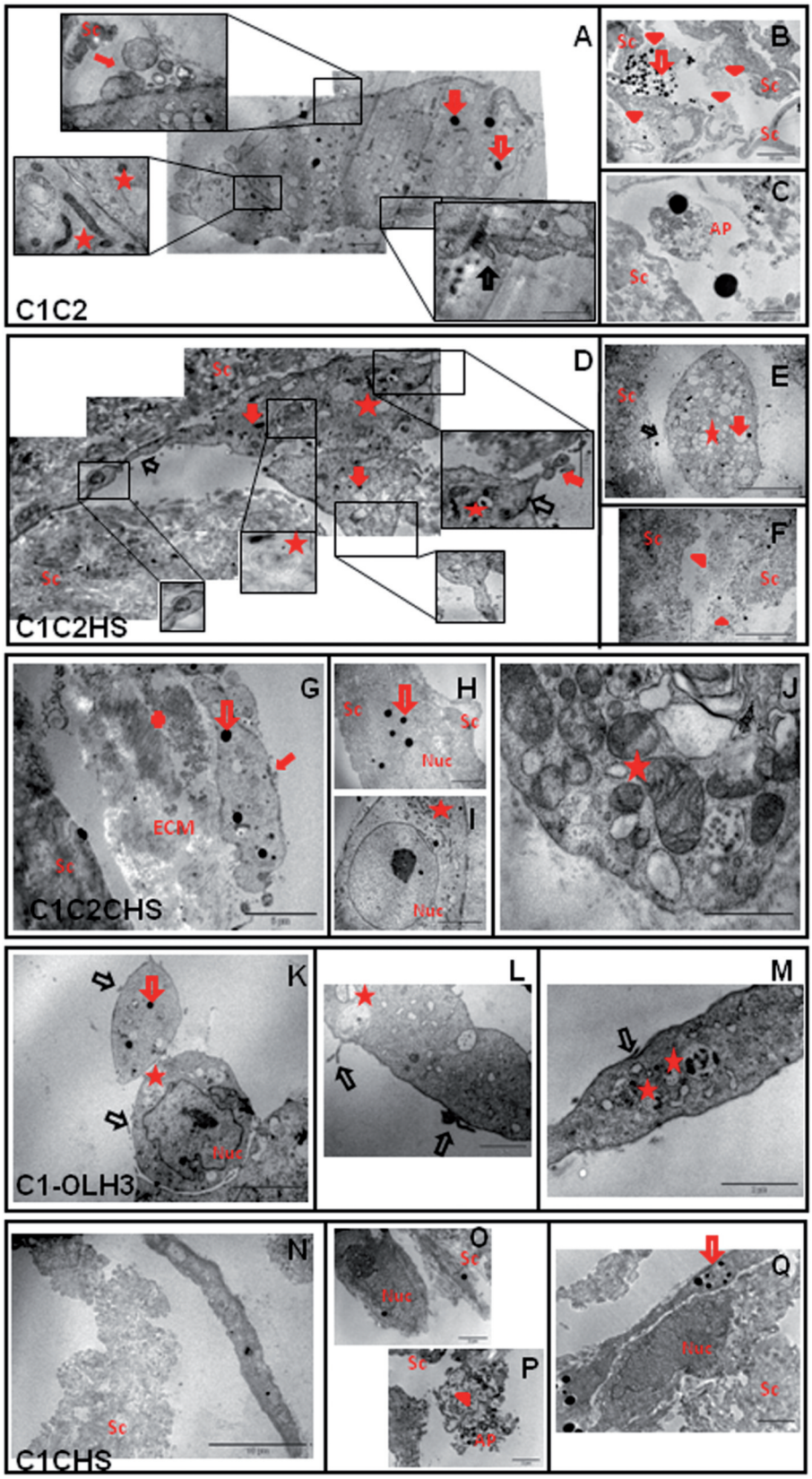


Fig. 11. Images obtained by transmission electron microscopy (TEM) from the different biomaterial constructs. The different scaffolds are shown from top to bottom. AP: highly vacuolated apoptotic cells; arrowheads: cellular debris; cross: fibers of collagen; empty black arrow: prolongations; empty red arrow: electron-dense granules; Sc: fibers of biomaterial; Nuc: nucleus; ECM: extracellular matrix. Scale bars: A) 2 μm (the main image) and 1 μm, 500 nm and 1 μm (details from upper-left to lower-right, respectively); D) 2 μm (the main image) and 500 nm, 500 nm, 2 μm and 2 μm (details from lower-left to upper-right); B, E, F, N, 10 μm; G, H, I, K, 5 μm; C, L, M, O, P, Q, 2 μm; J, 1 μm.

CD73 and CD105. In this study even when CD73 and CD105 are not >95% positive, these cells fulfills all the other requirements (positive for CD90, not positive for CD34 and CD45 and capacity for multipotent differentiation). This difference could be due to the age of donors (74.66±10.25 and 76.25±2.87) and that all of them presented severe OA (hip replacements because of stage 4 -OA). In our previous works, we found differences in the positivity of surface markers between healthy and OA cells (from different joint tissues) (Hermida-Gómez et al., 2011; Cicione et al., 2013).

BMSCs can be induced toward chondrogenic differentiation for cartilage tissue engineering purposes in a variety of 3D culture environments (Markway et al., 2010; Ragetly et al., 2010), facilitated by administering TGFβ (Lisignoli et al., 2005; Hu et al., 2009; Ragetly et al., 2010). *In vivo*, growth factors of the TGFβ superfamily are effective for inducing chondrogenesis (Hunziker, 2002). In our study, the commercial chondrogenic medium included TGFβ-3.

Several studies have obtained a cartilage-like ECM and cell phenotype using chondrocytes and cartilage-derived matrix (Cheng et al., 2011), Col I (De Franceschi et al., 2005; Negri et al., 2007), fibrin glue gel (Scotti et al., 2010), Col I and Col III (Ehlers et al., 1999) and hyaluronan benzyl-ester scaffolds (Grigolo, et al., 2002b). Other studies have obtained satisfying results with MSCs using nanofibrous poly L-lactic acid (Hu et al., 2009), chitosan (Ragetly et al., 2010), alginate and hyaluronan (Tritz-Schiavi et al., 2010) and hyaluronan benzyl-ester scaffolds (Lisignoli et al., 2005). In our study we chose biomaterials consisting of typical cartilage ECM compounds: Col and PGs or GAGs from natural sources.

The use of natural compounds as biomaterials provides an advantage over semi-synthetic scaffolds because of their limited intrinsic bioactivity (Schagemann et al., 2013). Col is a biodegradable material that can be reconstituted into fibrous structures simulating the native ECM in tissues (Chan and Leong, 2008), like the sponges that we used in this work. The use of biomaterials that mimic native ECM should be advantageous in *in vivo* studies. Xenogenic natural biomaterials could cause immunological rejection when implanted (Tuli et al., 2003; Chan and Leong, 2008), but Col possesses good biocompatibility (Chan and Leong, 2008) and cytocompatibility (Xu et al., 2012). Moreover, the use of xenogenic Col avoids viral contamination (De Franceschi et al., 2005) and its degradation produces non-toxic products (Hunziker, 2002). On the other hand, the use of CHS in biomaterials promotes proliferation (Ko et al., 2009), regulates cartilage-specific genes and the expression of the chondrocyte phenotype and prevents MSCs from further differentiating into a hypertrophic phenotype (Lee et al., 2006; Varghese et al., 2008; Kagita et al., 2010). Scaffolds for tissue engineering should be capable of facilitating infiltration, attachment, proliferation and cell differentiation (Tuli et al., 2003). Wang et al., (2012) suggested that biomaterials composed of Col I improve hydrophilicity of the scaffold and allow better cell adhesion, proliferation activity and differentiation. In our experiments, hBMSCs showed improved capacity for cell attachment and synthesis of ECM in biomaterials composed of both Col I and II (C1C2, C1C2HS, C1C2CHS) than in biomaterials composed only of Col I (C1-OLH3, C1CHS), which had larger pore size. Scaffolds should be porous enough for efficient nutrient and metabolite transport (Negri et al., 2007; Chan and Leong, 2008; Kock et al., 2012). Several studies have found inhomogeneous proliferation, ECM deposition or differentiation between central and peripheral parts of scaffolds (Lee et al., 2006; Markway et al., 2010; Ragetly et al., 2010; Scotti et al., 2010; Zhang et al., 2012) when scaffold porosity was unsuitable. Indeed, we observed homogeneous growth only in biomaterials composed of both types of Col (C1C2, C1C2HS, C1C2CHS) and that BMSCs could easily infiltrate, attach, proliferate and synthesize ECM in these scaffolds.

We observed COL II in C1C2, C1C2HS and C1C2CHS, with COL I being downregulated only in C1C2CHS. Increased COL I expression has often been seen to be closely related to chondrogenic differentiation of MSCs *in vitro* and to pre-chondrogenic MSCs (Grigolo, et al., 2002a; Lopez-Ruiz et al., 2013). The same phenomenon occurs with Col X, whose expression increases during *in vitro* MSC culture (Kock et al., 2012). The presence of both Col I and X may indicate the presence of undifferentiated MSCs inside the construct, mixed with differentiated chondrocyte-like cells. It may be that during chondrogenesis cells do not stop proliferating at the time of cell differentiation (Lisignoli et al., 2005). The highest presence of Col I was observed in C1C2HS, suggesting that the cells were in an early state of differentiation.

Both immunohistochemistry and molecular biology confirmed higher expression of Col X on C1C2 than in biomaterials composed of HS, CHS or heparin. Several authors found that the use of CHS for cartilage engineering prevents hypertrophy (Chen et al., 2015). On the other hand, chondrogenic hypertrophy of MSC could be avoided, with specific strategies at protein/gene level, when cultured in expansion or chondrogenic differentiation medium (Chen et al., 2015). For example, using FGF2 and WNT3A in MSC expansion medium in combination with WNT inhibition during differentiation (Narcisi et al., 2015), using specific PTHrP isoforms in chondrogenic differentiation medium (Lee and Im, 2012) or via TGFβ/Smad signaling modulation (Hellingman et al., 2011).

The early state of differentiation is also in agreement with SEM and TEM analyses in which we could see cells with rounded/oval shapes (Tew et al., 2008; Hu et al., 2009; Cheng et al., 2011) and many mitochondria, similar to mature chondrocytes (Fuss et al., 2000; Negri et al., 2007), as well as flattened cells resembling MSCs. The structure and composition of articular cartilage

varies according to its distance from the surface, and chondrocytes from the different zones differ in size, shape and metabolic activity (Newman, 1998). Cartilage electron microscopy studies (Poole et al., 1987) showed chondrocytes with a flattened discoid shape and no clear evidence of a pericellular capsule in superficial layers of cartilage. In the C1C2CHS constructs that we tested, we observed Col horizontal fibers by TEM, similar to the superficial zone of cartilage (Khoshgoftar et al., 2011). The presence of some undifferentiated MSCs or chondrocyte-like cells without a depth-dependent organization could be an asset for cartilage engineering, because an immature engineered neotissue is able to mature into normal tissue when implanted into a joint (Pabbruwe et al., 2009). Because cellular phenotype and ECM vary in the cartilage zones (Newman, 1998), the constructs, once implanted, influence the metabolism of host cells (Negri et al., 2007), and the quality of the tissue-engineered cartilage (Fuentes-Boquete et al., 2008). Also, the movement and mechanical loads of the host joint are essential for development and maintenance of normal articular cartilage structure (Khoshgoftar et al., 2011). Continuous passive motion has been reported to encourage healing of full-thickness defects, and the regenerated tissue closely resembles hyaline cartilage (Newman, 1998). Furthermore, tissue engineering studies using depth-dependent scaffold properties or cell sources to create a depth-dependent structure have shown limited success (Khoshgoftar et al., 2011).

Col II is usually used as a marker for hyaline cartilage (Negri et al., 2007) and a PG-rich ECM is also a hallmark of chondrogenesis (Tew et al., 2008). Many tissue engineering methodologies produce cartilage-like neotissues with macromolecular components similar to those of native cartilage ECM. A major challenge is to produce constructs having biochemical, biomechanical and structural properties functionally equivalent to *in vivo* cartilage (Lee et al., 2010b).

The expression of Agg varies widely between individual patients, suggesting that this PG is unsuitable as a marker for chondrocyte differentiation (Grigolo, et al., 2002b). Although C1C2, C1C2HS and C1C2CHS were strongly positive for PGs in our study, we did not find Agg expression at the protein level, although it was present at the gene level.

Several studies suggest that adding exogenous Col II to MSCs culture is better than adding Col I. Chen et al. (2005) concluded that Col II in the culture medium allows GAG synthesis but Col I decreases the Agg, Sox 9 and Col II levels. Furthermore, Wong et al. (2010) found more deposition of GAG in chondrocyte cultures, in response to addition of Col II to the culture medium. In this study, the hBMSCs on biomaterials composed of Col II presented better GAG synthesis than hBMSCs on Col I biomaterials. However, the presence of Col I in these biomaterials can be downregulating the Agg expression, whereas Sox 9 and Col II is not affected.

Scaffold pore size is also an important factor in preventing the newly synthesized proteins and PGs from escaping (Mouw et al., 2005). The larger amounts of secreted-Col detected in C1-OLH3 and C1CHS culture supernatants may be explained by this phenomenon. Several studies have found that the use of CHS in biomaterials promotes the secretion of Col II (He et al., 2012); this agrees with the results of our Col assays in which we found more peaks of total Col released in CHS biomaterials than in the others. We found two peaks of released Col concentration in biomaterials composed of CHS, but only one in the other biomaterials. Finding the first peak in CHS biomaterials at the beginning of culture indicates that CHS provokes an immediate secretion of ECM components. Histologically, we found Col II in the ECM from C1C2, C1C2HS and C1C2CHS constructs. Col I was detected in these constructs, but at lower levels in constructs of biomaterials composed of CHS (C1C2CHS).

In summary, C1C2HS showed the highest histological staining values for PCNA (proliferation), SO and Col II, indicating that this biomaterial allows better proliferation and attainment of improved chondrogenic-ECM properties. Furthermore, C1C2HS showed the highest values for Col I, indicating the presence of MSCs, and a negligible amount of VK, indicating absence of mineralization. C1C2CHS had a higher intensity value for AB than C1C2HS, but was lower in SO and PCNA. It also had lower staining intensity than C1C2HS for VK, but higher for MMP-13, indicating the onset of hypertrophy. In general, C1C2CHS showed intermediate values, indicating a desirable early state of chondrogenic differentiation. C1C2 had a lower percentage of cells than C1C2HS and C1C2CHS and also showed less proliferation (PCNA) than C1C2HS but more than C1C2CHS. Otherwise, C1C2 presented intermediate values for chondrogenesis, de-differentiation and hypertrophy (Col X). C1-OLH3 also had lower values for proliferation (PCNA) and chondrogenesis (AB, SO, Col II) and did not have de-differentiated or hypertrophic characteristics. In C1CHS, the percentage of cells was so low that the values were not quantifiable.

In conclusion, our results show that biomaterials composed of both Col I and II allow growth and proliferation of hBMSCs throughout the scaffold. hBMSCs cultured in chondrogenic medium with TGFβ-3 in these biomaterials were able to initiate chondrocyte-like differentiation at an early state and to synthesize a cartilage-like ECM. Of all constructs tested in this study, C1C2HS and C1C2CHS presented better cartilage-like phenotype than the other ones, because of the expression of chondrogenic genes, deposition of GAGs and Col II in the ECM, and the lack of hypertrophy. Further studies are needed to shed light on the suitability of these biomaterials for tissue engineering. In an *in vitro* model of cartilage repair we will attempt to ascertain whether these constructs are capable of integration with surrounding host tissue and the optimal level of construct maturity for implantation. *In vivo* animal models are also needed to test the biochemical and

biomechanical characteristics of the neo-cartilage.

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