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Mesenchymal stromal cell-derived extracellular matrix influences gene expression of chondrocytes

Shraddha Thakkar, Corina A Ghebes, Maqsood Ahmed, Cindy Kelder, Clemens A van Blitterswijk, Daniel Saris, Hugo A M Fernandes and Lorenzo Moroni

Tissue Regeneration Department, University of Twente, Drienerlolaan 5, 7522 NB Enschede, The Netherlands

E-mail: l.moroni@utwente.nl

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Abstract

Decellularized extracellular matrix (ECM) has recently gained a lot of interest as an instructive biomaterial for regenerative medicine applications. In this study, the ability of adult human mesenchymal stem cell (hMSC)-derived ECM to rescue the phenotype of osteoarthritic (OA) chondrocytes and to further stimulate the differentiation of healthy (HL) chondrocytes was evaluated. ECMs were prepared by decellularizing hMSCs cultured in basic medium (BM) and chondrogenic medium (CM). The obtained ECM was then combined with a polymeric solution of Poly (ε-caprolactone) (PCL) dissolved in 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP) and electrospun meshes were fabricated. Electrospun ECM scaffolds were characterized using scanning electron microscopy (SEM) and picrosirius red staining was used to confirm the presence of collagen. OA and HL chondrocytes were cultured on scaffolds containing hMSC ECM in BM or CM and compared to PCL electrospun scaffolds without ECM. Metabolic activity and chondrogenic gene expression were assessed by Alamar blue assay and quantitative PCR (qPCR) analysis, respectively. The ECM presence resulted in a significant difference in chondrocyte metabolic activity compared to PCL scaffolds alone. HL chondrocytes cultured for 21 days in chondrogenic medium on electrospun scaffolds containing hMSC ECM from BM showed a significant increase in collagen II and aggrecan expression compared to hMSC ECM from CM and PCL scaffolds without ECM incorporation. No significant influence of hMSC ECM presence on the chondrogenic signature of OA chondrocytes was found. The influence of decellularized hMSC ECM on HL chondrocytes suggests that hMSC-derived ECM scaffolds are promising candidates for cartilage tissue engineering applications.

S Online supplementary data available from stacks.iop.org/BF/5/025003/mmedia

(Some figures may appear in colour only in the online journal)

Introduction

Extracellular matrix (ECM) consists of a mixture of structural and functional molecules such as collagen, fibronectin, elastin, laminin, glycosaminoglycans and other glycosylated proteins organized in a unique three-dimensional (3D) pattern [1]. Diversity in the composition of ECM depends on the tissue source and its organization varies with location and physiological requirements [2]. The ECM provides structural and physical stability to the tissue and plays a key role in regulating cell signaling via receptors such as integrins [3]. ECM-mediated signaling is involved in a diverse range of cellular functions such as cell migration, proliferation, differentiation, immune response and wound healing [4–6]. Recently, biological scaffolds composed of ECM have been investigated for repair and replacement of tissues and organs

in tissue engineering (TE) applications [4, 7–11]. Matrigel (an animal-derived gelatinous protein mixture) supports attachment and differentiation of cells and is extensively used to assess the invasive potential of cancer cells [12]. However, this ECM is of animal origin and reports have also shown that it enhances tumorigenicity of a number of cells *in vivo*, limiting its use in clinical applications. Thus, there is a need to develop alternatives such as ECM scaffolds that can equally mimic the *in vivo* microenvironment without promoting tumorigenicity. As is difficult to mimic the *in vivo* ECM, researchers have tried to use the ECM in tissues and organs after a decellularization treatment. Some widely investigated decellularized tissues include heart [13], heart valves [14–17], blood vessels [18, 19], small intestinal sub mucosa (SIS) [20, 21] and liver [22, 23]. Kasimir *et al* used a decellularized porcine heart valve matrix seeded with endothelial cells to eliminate thrombogenicity in heart valves [24]. ECM scaffolds derived from porcine chondrocytes promoted chondrogenic differentiation of rabbit MSCs and also helped to maintain its phenotype *in vivo* [25]. Cheng *et al* cultured chondrocytes in collagen microspheres to deposit GAG-rich ECM. Upon decellularization, these microspheres supported chondrogenic differentiations of MSCs [26]. Furthermore, Flynn observed that decellularized adipose tissue supports angiogenesis when cultured with human adipose-derived stem cells, without the need for exogenous differentiation factors [27]. The promising results obtained from this tissue-specific derived ECM indicated it as an excellent scaffold material, which eventually resulted in its commercialization [28–30].

More recent studies also suggested that decellularized matrices derived from hMSCs maintained a stem cell niche and promoted MSCs proliferation while retaining their stemness [31, 32]. Researchers have also used cell-derived ECM to modify the surface of biomaterials to improve cell–material interactions. Datta *et al* coated titanium meshes with MSCs and decellularized them after deposition of bone-like ECM on the metal. The constructs were re-seeded with rat MSCs resulting in enhanced calcium deposition compared to constructs without ECM, thus showing ECM-enhanced osteogenic differentiation of MSCs [33]. Interestingly, Postovit *et al* showed that ECM derived from embryonic stem cells changed the gene expression profile of melanoma cells to a phenotype characteristic of normal pigment cells, indicating the ability of ECM to reprogram cells [34].

Similarly, we hypothesize that ECM derived from adult hMSCs may have the potential to rescue the phenotype of osteoarthritic chondrocytes and enhance the differentiation of healthy chondrocytes. Degeneration of articular cartilage eventually leads to the development of osteoarthritis (OA) [35–37]. At a cellular level, cartilage cells lose their round morphology and show a shift in collagen distribution. While in healthy articular cartilage chondrocytes produce predominantly collagen type II and its associated collagen types IX and XI, in OA a switch toward the secretion of collagen type I is observed with a concomitant decrease in the expression of collagen II and aggrecan [38]. Ongoing research in this field focuses on preventing further damage and repairing the damaged cartilage using tissue engineering strategies [39–41]. In cartilage tissue engineering, dedifferentiation of chondrocytes during *in vitro* expansion culture is, however, still a major barrier [42]. To facilitate cartilage formation *in vitro*, one needs to provide a 3D environment with ample possibilities for cell–cell contact and supply of nutrients, while maintaining a native chondrogenic phenotype.

Despite the enhanced bioactivity of decellularized ECM, its use as a biomaterial for the regeneration of hard tissues is still limited by insufficient mechanical properties. Certain processing steps, such as cross-linking, are still required in order to improve the mechanical properties of the constructs [43]. Alternatively, combining decellularized ECM with synthetic scaffolds may result in enhanced mechanical properties and help in the process of chondrogenesis while preventing adverse effects on the biological activity of the ECM. Synthetic scaffolds with micrometric and nanometric features can be reproducibly fabricated, for example, by electrospinning, with tailored mechanical properties and may mimic artificially the physical and chemical properties of the native ECM. Yet, most of these 'synthetic ECMs' lack biological activity to better interact with the cells. Therefore, in this study, we aimed at evaluating the influence of electrospun hybrid scaffolds resulting from a combination of hMSCs-derived ECM and PCL as instructive 'hybrid' microenvironments on the fate of OA and HL chondrocytes.

Material and methods

Cell culturing

Both hMSCs and chondrocytes were obtained from human donor after written informed consent in conformity with National Dutch laws. hMSCs (female, 75 years old) were isolated from the bone marrow and proliferated as described previously [44]. Briefly, aspirates were suspended by using 20-gauge needles, plated at a density of 5×10^5 cells cm^{-2} and cultured in hMSC proliferation medium containing α-minimal essential medium (Life Technologies), 10% fetal bovine serum (Cambrex), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 units mL^{-1} penicillin (Life Technologies), 10 μg mL^{-1} streptomycin (Life Technologies) and 1 ng mL^{-1} basic fibroblast growth factor-2 (FGF-2) (Instruchemie). Cells were grown at 37 °C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week, and cells were used for further sub-culturing or cryopreservation.

hMSCs between passage 2 and 4 were seeded in basic medium (BM) and in chondrogenic medium (CM) to obtain extracellular matrix (ECM), which were later used for scaffold fabrication. Cells were cultured in a tissue culture flask at a seeding density of 5000 cells cm^{-2} for 3 weeks. BM consisted of the above-mentioned proliferation medium without FGF-2. CM consisted of Dulbecco's Modified Eagle Medium (DMEM, Sigma), Glutamax (100X, Sigma), 0.2 mM ascorbic acid (Sigma), 100 units mL^{-1} penicillin (Gibco), 100 μg mL^{-1} streptomycin (Gibco), 0.4 mM

Proline (Sigma-Aldrich), 100 μg mL^{-1} sodium pyruvate (Gibco), 50 μg mL^{-1} insulin transferrin selenium-premix, 10 ng mL^{-1} transforming growth factor beta3 (TGF-β3) (R&D systems, Abingdon, United Kindgom) and 1 $\times$ 10^{-7} M Dexamethasone (DEX) (Sigma). TGF-β3 and DEX were added to the chondrogenic medium shortly before use. The medium was initially refreshed after 10 days and then every 4 days during subsequent weeks. Upon confluence the cells secrete ECM which was subsequently isolated by decellularization.

OA and HL chondrocytes (passage P0) were obtained from donors (27 and 44 years old) who had undergone knee surgery. The nucleated cells were isolated as described previously [45]. Briefly, dissected cartilage was incubated for 20–22 h in collagenase type II solution containing 0.15% collagenase (Worthington), Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). The suspension was filtered through a 100 μm mesh nylon filter (cell strainer Nucleon) and cells were washed two times with PBS supplemented with penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). The cells were plated at a density of 5000 cells cm^{-2} and cultured for 3 weeks in proliferation medium containing DMEM supplemented with 10% fetal bovine serum, 1 $\times$ non-essential amino acids (Sigma-Aldrich), 10 mM HEPES buffer (Biowhitaker), 0.2 mM ascorbic acid 2-phosphate (InVitrogen), 0.4 mM proline (Sigma-Aldrich), 100 U mL^{-1} penicillin (Invitrogen) and 100 μg mL^{-1} streptomycin (Invitrogen). Media was changed every 3 days and constructs were cultured in an incubator at 37 °C and 5% CO_2.

Decellularization of ECM

The decellularization protocol for ECM isolation involved addition of 20 mM ammonium hydroxide (NH_4OH) for 3–4 min [46]. NH_4OH disrupts the cell membrane leaving an intact ECM. Upon confirmation of complete cell lysis under a microscope, distilled water (Gibco) was added to dilute the NH_4OH, wash and remove the remaining cell debris. After carefully aspirating the diluted NH_4OH solution, 10 units mL^{-1} of Deoxyribonuclease 1 (Invitrogen) was added and incubated for 30 min to cleave the DNA. Upon extensive washing a thin layer of ECM was attached to the bottom of the flask. This layer was carefully detached using a cell scraper. The ECM thus obtained had a transparent jelly-like structure and was used for electrospinning together with a polymer solution.

Material for electrospinning

Poly (ε-caprolactone) (PCL) was used as the host polymer for electrospinning. In order to obtain a viscous polymeric solution which would aid fiber formation during electrospinning, PCL with a molecular weight of 42 500 was acquired from Sigma-Aldrich. 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP) was used as a solvent for PCL [47]. HFIP (99.9%) was purchased from Biosolve.

Lyophilization of ECM for electrospinning

Two types of ECM were used for scaffold fabrication using electrospinning: ECM obtained from hMSC cultured in BM (hMSC ECM) and from hMSC cultured in CM (hMSC ch ECM). The ECM was lyophilized by freeze drying to remove water from the matrix, which helps to prevent its degradation. Briefly, the samples were frozen in liquid nitrogen and placed in a freeze drying vessel, which was attached to the freeze-dryer (Labconco, Beun de Ronde).

Preparations of solutions for electrospinning

A 20% wt/v solution of PCL in HFIP was prepared. The solution was sealed and kept on a magnetic stirrer overnight at room temperature. Polymeric solutions including ECM were prepared by adding to the 20% wt/v of PCL solution a calculated amount of lyophilized ECM. For each condition, 125 μg protein mL^{-1} HFIP (0.0125% wt/v) was added to the solutions. Solutions were made using hMSC ECM and hMSC ch ECM. These solutions were kept on a magnetic stirrer for 8 h for homogenous dissolution of ECM before spinning.

Electrospinning procedure

The procedure for scaffold fabrication via electrospinning involves spinning of a polymer solution to form micro- or nanometer range fibers, which can be collected to form an electrospun mesh [48]. For all experiments, a flow rate of 2.5 mL h^{-1} was used. The syringe was connected to a capillary tube, whose other end was attached to a metallic needle (0.8 mm diameter), clamped and held at a distance of 20 cm from the collector. One end of the power supply was attached to the metallic needle, while the collector or the aluminum foil was grounded. A potential difference of 15 kV was applied between the needle and the collector that generated an electric field at the tip of the needle. The temperature was kept constant at 20 °C and humidity varied between 33% and 35%, respectively. Polymeric solutions with ECM were electrospun in a similar manner.

Characterization of electrospun scaffolds

Scanning electron microscope. Scanning electron microscopy (SEM) was used to observe the structure of the fabricated scaffolds. Prior to SEM analysis, samples were mounted on a holder and sputtered with gold using a Cressington sputter coater. SEM images were taken at different magnification using a 10 kV beam. The obtained images were used to analyze and quantify the diameter of the electrospun fiber. The fiber diameters were calculated by measuring 20 fibers per condition using Image J (Image J 1.43o8, National Institutes of Health, USA).

Picrosirius red staining. Picrosirius red staining is a histological method to analyze the presence and spatial distribution of collagen fibers, specifically collagen type I and III [49]. The staining was done following the manufacturer's protocol (Polysciences, Inc.). Prior to the staining, the samples

Table 1. Forward and reverse primers for gene expression analysis using RT-PCR.

Gene	Forward Primer (5′- > 3′)	Reverse primer (5′ - > 3′)
Collagen I	GTCACCCACCGACCAAGAAACC	AAGTCCAGGCTGTCCAGGGATG
Collagen II	CGTCCAGATGACCTTCCTACG	TGAGCAGGGCCTTCTTGAG
Collagen X	GCAACTAAGGGCCTCAATGG	CTCAGGCATGACTGCTTGAC
Aggrecan	AGGCAGCGTGATCCTTACC	GGCCTCTCCAGTCTCATTCTC
Sox9	TGGGCAAGCTCTGGAGACTTC	ATCCGGGTGGTCCTTCTTGTG
GAPDH	CGCTCTCTGCTCCTCCTGTT	CCATGGTGTCTGAGCGATGT

were washed twice with PBS. Briefly, the electrospun scaffolds were placed in solution A for 2 min and washed with distilled water. Next, samples were placed in solution B for 110 min. Lastly the scaffolds were immersed in solution C for 2 min. The samples were dehydrated by immersing in 70% ethanol for 45 s, dried after peeling off the aluminum foil, mounted on a glass slide and analyzed under a polarized light microscope.

Cell seeding on electrospun scaffolds. PCL electrospun disks with a diameter of 15 mm were obtained from electrospun mesh fabricated from PCL-HFIP solutions with and without ECM. The disks were soaked in 100% ethanol for 30 min, dried in the flow cabinet for 15 min, washed twice with PBS and transferred to 24 non-culture-treated well-plates. O-rings were used to secure the scaffolds in place. Next, scaffolds were soaked in chondrocyte BM and incubated for 4 h. Fifty thousand cells per scaffolds were seeded in 50 μl of BM ($n = 6$) and incubated for 1 h at 37 °C and 5% CO_2, to allow cell attachment to the scaffolds. Finally, 1 mL of BM and CM, respectively, was added to each well.

Alamar blue assay for metabolic activity. Cell metabolism was assessed using Alamar blue (AB) assay according to the manufacturer's protocol [50]. Briefly, the culture medium was replaced with a medium containing 10% (v/v) AB (Bioscience, Camarillo, CA, USA) solution and cells were incubated for 4 h. Fluorescence was measured by spectrometry (Victor3, Perkin-Elmer, USA) at a wavelength of 590 nm. Cell metabolism was analyzed at days 10, 14, 17 and 21 and measured values were corrected with blank.

RNA isolation and gene expression analysis using quantitative PCR. To analyze the expression of chondrogenic markers of OA and HL cells seeded on electrospun scaffolds with and without ECM, the total RNA was isolated using Trizol (Invitrogen) according to manufacturer's protocol. Two samples from each scaffold were combined to ensure sufficient quantity of RNA. Briefly, the samples were lysed by adding Trizol. After addition of chloroform and centrifugation, the aqueous phase containing the RNA was carefully collected. Isolation of RNA proceeded using an RNA isolation kit (Macherey-Nagel) as per manufacturer's instructions. RNA was precipitated using isopropyl alcohol, washed with 75% ethanol and re-suspended in RNA free water. The collected RNA was quantified using a Nanodrop. After quantification, cDNA was synthesized from 200 ng of RNA using an i-script cDNA synthesis kit (Bio-Rad) in an MJ mini gradient Thermal cycler (Bio-Rad), as per manufacturer's instructions. One μL of cDNA (16 ng μL^{-1}) was used to perform the qPCR. Table 1 shows the primer sequence used for qPCR analysis. Data were analyzed using Bio-Rad software by adjusting the noise band to the exponential phase. Fold induction was calculated using the Delta Ct method upon normalization with GAPDH [51].

Statistical analysis

Data are presented as mean and standard deviation. Quantitative assays were performed with six samples ($n = 6$) unless specified. A two-way ANOVA followed by a Tukey's test was performed for metabolic activity data, while a one-way ANOVA followed by a Tukey's test was performed for PCR analysis.

Results

ECM structure analysis and characterization

hMSCs were cultured in BM and CM medium for 21 days to obtain ECM. After decellularization, the freeze-dried ECM structure was observed under SEM (figure S1, supplementary information, available at stacks.iop.org/BF/5/025003/mmedia). The structure of hMSC ch ECM looked crystalline, demonstrating fused clusters or aggregates of proteins, distinctively different from hMSC ECM.

Electrospun scaffold characterization

Lyophilized hMSC ECM were incorporated in PCL solution and electrospun to form micro- and nano-meter fibrous mesh. PCL scaffold without ECM was used as a control. Scaffolds comprised a bimodal fiber population in the micro and nanoscales. The mean fiber diameter of electrospun PCL without ECM was 1.73 $\pm$ 0.51 μm in the micrometer range and 180 $\pm$ 0.04 nm in the nanometer range. In the case of electrospun PCL scaffolds including hMSCs ECM, fibers measured 1.29 $\pm$ 0.39 μm in the micrometer range and 180 $\pm$ 0.04 nm in the nanometer range. In the case of electrospun PCL scaffolds including hMSCs ch ECM, the fiber diameter was 1.23 $\pm$ 0.35 and 150 $\pm$ 0.04 nm. Our results showed that the addition of ECM leads to a slight decrease in the diameter of micrometric fibers and of the nanometric fibers in the case of hMSCs ch ECM when compared to the control. The fiber structure and orientation of the electrospun scaffolds were observed using SEM (figure 1). It can be seen that all the scaffolds are porous in nature and closely mimic the structure of native ECM.

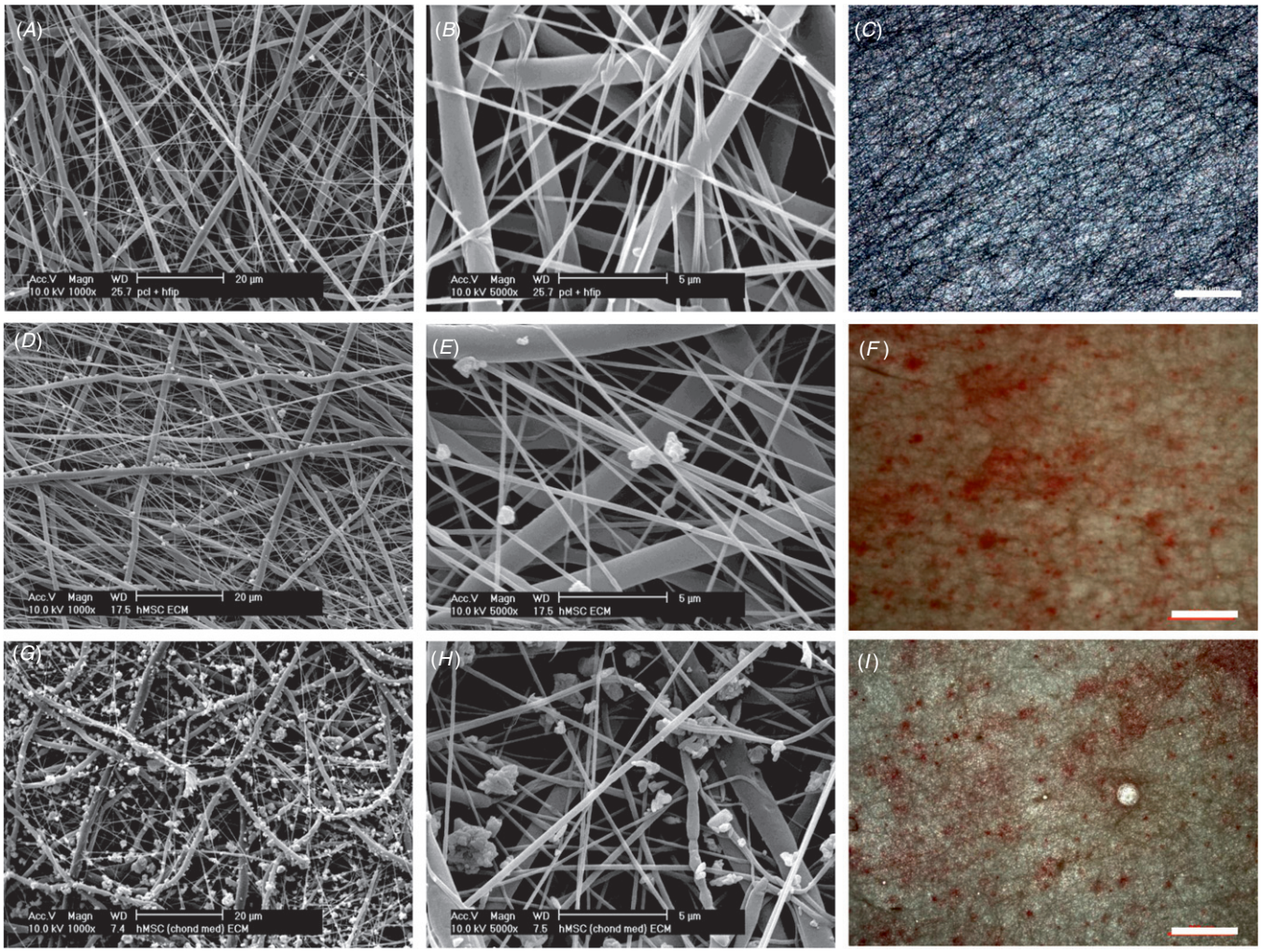

Figure 1. SEM images of electrospun scaffolds with and without ECM (*A*), (*B*), (*D*), (*E*), (*G*), (*H*). PCL electrospun scaffolds without ECM (*A*)–(*C*). PCL electrospun scaffolds with hMSC ECM (*D*)–(*F*). PCL electrospun scaffolds with hMSC ch ECM (*G*)–(*I*). Picosirius red staining (40X) showing the presence of collagen in ECM containing PCL electrospun scaffolds (*F*) and (*I*) and its absence in blank PCL scaffolds (*C*). Magnification: 1000 × (*A*), (*D*), (*G*), 5000 × (*B*), (*E*), (*H*), 40 × (*C*), (*F*), (*I*); scale bar: 200 μm (40 ×), 20 μm (1000 ×), 5 μm (5000 ×).

Picrosirius red staining was carried out to confirm the presence of ECM in the electrospun scaffolds. Figure 1 shows that electrospun ECM scaffolds stain positive with picrosirius red staining validating the presence and distribution of collagen, hence ECM in the scaffolds. Although equal amount of ECM was added to the solutions used for electrospinning, variation in staining intensity was observed. hMSC ECM scaffold showed more intense staining compared to hMSC ch ECM scaffolds. As expected, no collagen was observed in PCL scaffolds without addition of ECM.

Influence of electrospun ECM on metabolic activity

Metabolic activity of OA and HL chondrocytes was measured by Alamar blue assay on days 10, 14, 17 and 21. Figure 2 shows the comparison of metabolic activity based on the cell type and the medium used. For the initial 2 weeks, a statistically significant increase in metabolic activity in time was observed in all conditions. After day 14, both OA and HL chondrocytes reached a plateau. OA chondrocytes cultured in BM demonstrated a drop in metabolic activity, though not statistically significant. A significant increase in metabolic activity was found for both OA and HL chondrocytes cultured in hMSC ch ECM in BM at day 17. The metabolic activity of HL cells was significantly higher in scaffolds containing hMSC ECM and hMSC ch ECM compared to PCL alone at day 21. When cells were cultured in CM media, no significant difference was found among the different scaffolds with the exception of OA chondrocytes cultured on scaffolds containing hMSC ECM.

Gene expression analysis

qPCR analysis was performed after 21 days on OA and HL chondrocytes cultured on electrospun ECM scaffolds. A panel of chondrogenic markers (Sox 9, Aggrecan, Collagen type I, II and X) was analyzed. OA cells seeded on hMSC ECM scaffold and cultured in CM for 21 days (figure 3) showed an increase in collagen X compared to the other scaffold conditions, though not statistically significant. Cells in CM

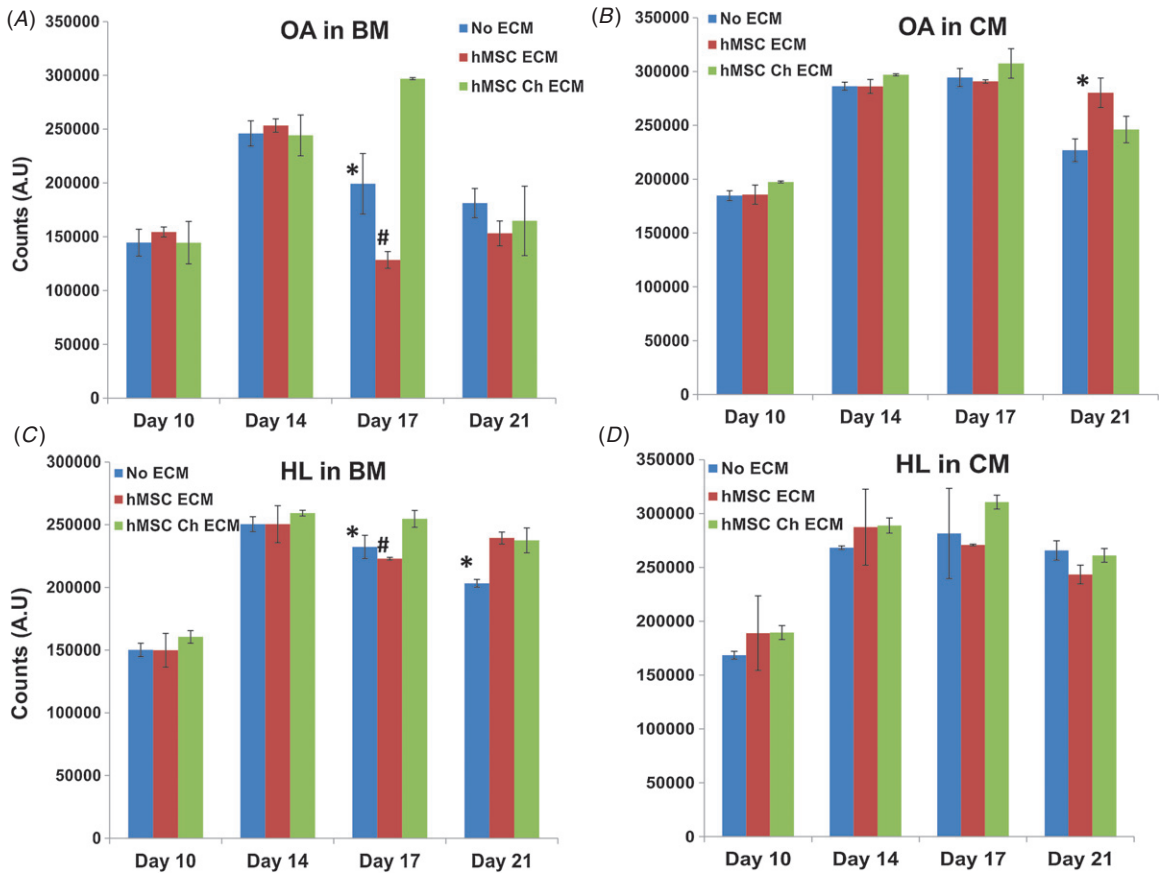

Figure 2. Metabolic activity of OA (*A*), (*B*) and HL (*C*), (*D*) cells cultured on scaffolds with and without ECM in BM (*A*), (*C*) and CM (*B*), (*D*). Metabolic activity was measured on days 10, 14, 17 and 21 using Alamar blue assay. * and # show statistical differences with respect of other groups at the same time point ($p < 0.05$).

demonstrated a significantly higher collagen X expression than those in BM. OA chondrocytes cultured on hMSC ECM in CM showed an increase in sox 9 and aggrecan (figure 3 and figure S2, supplementary information, available at stacks.iop.org/BF/5/025003/mmedia) compared to OA cells cultured on other scaffolds. However, the observed increase in expression was not statistically significant. A significant increase in aggrecan was observed when HL chondrocytes were cultured on hMSC ECM in CM compared to BM and to other scaffolds conditions in CM (figure 4). OA and HL chondrocytes cultured in CM showed a statistically significant increase in collagen type I and type II compared to those cultured in BM on hMSC ECM scaffolds. OA cells also showed a significant increase in collagen type I and type II when cultured on PCL scaffolds with no ECM in CM compared to BM. HL chondrocytes seeded on hMSC ECM in CM also showed a significant increase in the expression of collagen type II with respect to the other scaffolds.

Discussion

ECM is a complex network, composed of many kinds of molecules like collagens, fibronectin and proteoglycans. Owing to its unique 3D structure, mechanical properties, signaling potential and ability to regulate cell functions, ECM is gaining lot of attention as a biological scaffold material.

Isolated ECM components such as collagen elastin, laminin and fibronectin have been used for many applications [52–55]. However, these scaffolds fail to mimic the *in vivo* ECM, which contains a complex mixture of proteins which is difficult to recreate *in vitro*. Many studies have focused on decellularization of tissues and organs [13, 56–58]. Xenogenic and allogenic tissues are frequently used due to their availability; however, they suffer from problems associated with inflammatory and immunological reactions [59, 60]. An alternative approach could be using cell-derived ECM. This is obtained by culturing cells *in vitro* to deposit ECM, which can be used as a scaffold material upon decellularization. Many decellularization techniques have been developed to prepare cell-derived ECM as cell substrates [61]. Studies have been performed using cell-derived ECM scaffolds to evaluate the effect of the ECM on the cells cultured on the scaffold [62–64].

We extracted hMSC ECM by decellularization and further lyophilized it. According to the literature, ECM lyophilization leads to water removal and drying of the biologically active component contained in it, making the ECM proteins more stable [65]. Therefore, we assume that the obtained ECM was primarily composed of proteins. Moreover, the decellularization of the ECM was also reported by others to maintain the composition, mechanical integrity and biological activity of the ECM [66, 67]. Although

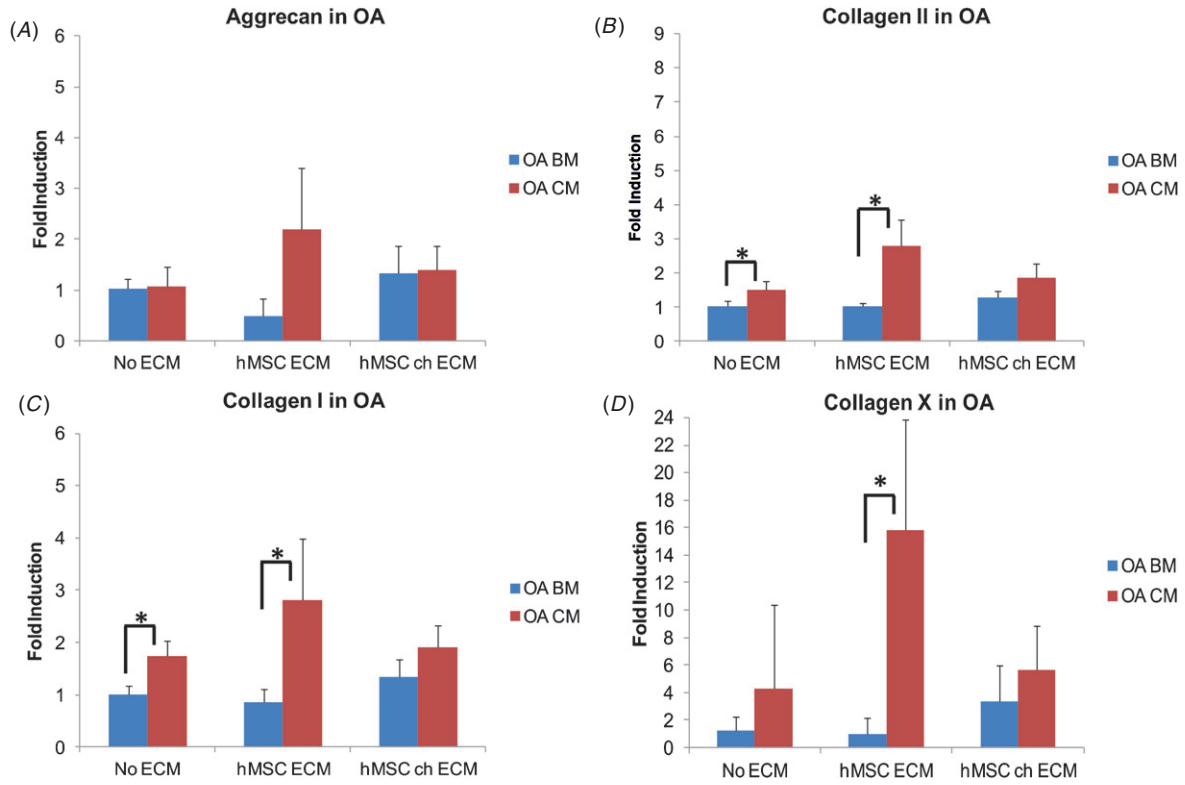

Figure 3. qPCR analysis of (*A*) aggrecan, (*B*) collagen II, (*C*) collagen I and (*D*) collagen X was performed after 21 days for OA cells culture on scaffolds fabricated with and without ECM. The fold induction was calculated relative to OA cells seeded on PCL scaffold without ECM in BM. * depicts statistical significance ($p < 0.05$).

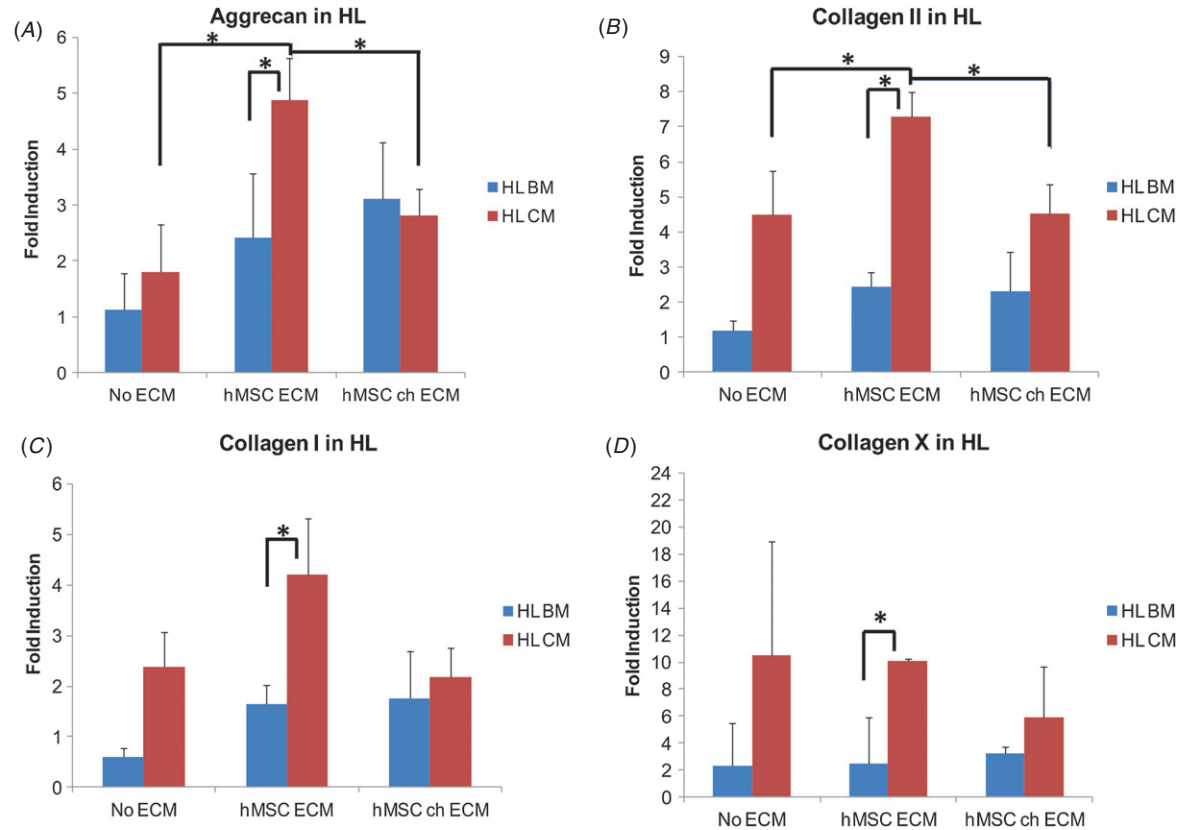

Figure 4. qPCR analysis of (*A*) aggrecan, (*B*) collagen II, (*C*) collagen I and (*D*) collagen X was performed after 21 days for HL cells culture on scaffolds fabricated with and without ECM. The fold induction was calculated relative to HL cells seeded on PCL scaffold without ECM in BM. * depicts statistical significance ($p < 0.05$).

we used the same seeding density to harvest ECM, the amount of ECM obtained was different in each case. Our result is in accordance with previous findings, where it was shown that the presence of morphogens increased proliferation of MSCs and induced cartilaginous ECM production [68–70]. Dissimilarities in terms of structure were also observed between ECMs in different culture media (figure S1, supplementary information, available at stacks.iop.org/BF/5/025003/mmedia). The discrepancy in morphology could be a consequence of differences in protein composition expressed by cells in different culture media. Also, it is known that the basic molecules that constitute the ECM may be similar in all organisms, and the distribution and organization of ECM molecules varies with tissue type, age of the host and species [71], explaining the non-static nature of ECM. This structural difference may have an effect on how the cells interact with the ECM scaffolds.

By using electrospinning, 3D scaffolds with high porosity and available surface were prepared. These structures closely mimicked the architecture of collagen in natural ECM. Scaffolds were fabricated by electrospinning the hMSC ECMs with PCL in an HFIP solution. Scaffolds spun without ECM were used as a reference. Figure 1 shows SEM images of scaffolds with ECMs displaying bead-like globular structures attached to the fibers, compared to the control scaffolds. It may be hypothesized that not all proteins were dissolved in HFIP, and the partially dissolved or un-dissolved proteins fused to the electrospun fibers, thereby leading to the observed bead-like globular structures. Zeugolis *et al* reported that electrospinning of ECM (collagen) out of HFIP dentures collagen, resulting in the creation of gelatin [72]. Additionally, ECM incorporating scaffolds consisted of less uniform matrix of fibers, showing high number of nanometer range fibers and reduced number of micrometer fibers (figure 1), although all electrospinning conditions were kept constant. The incorporation of ECM to the polymeric solution may have decreased the concentration of polymer, thus affecting the solution viscosity and the fiber size [73]. Another possible explanation could be related to the difference in source and concentration of collagen solution [74] observed by picrosirius staining (figure 1). Recent studies have shown that fiber diameter is an important parameter for cell culture in 3D, since cells sense and respond differently on fibrous scaffold with varying diameters [75]. For instance, Li *et al* showed that chondrocytes increased ECM production on nanofibers compared to micrometer fibers [76]. Collagen is the major component of ECM; its presence was evaluated in scaffolds through histological staining. PCL scaffolds without ECM showed negative picrosirius staining as expected. Scaffolds which had ECM incorporated within its fibers showed positive picrosirius red staining confirming the presence of collagen, and thus ECM (figure 1). Although the exact same amount of ECM was added in all polymeric solutions, the hMSC-ECM in BM scaffolds showed a more intense collagen staining compared to hMSC ch ECM scaffold. Additionally, the difference in collagen concentration in ECM may vary the structural properties of electrospun fibers [74]. Furthermore, from a comparison of picrosirius-stained scaffolds with its respective electrospun scaffolds, it can be observed that the bead-like globular structures on the SEM images of the fibers coincided with the aggregates of collagen present on picrosirius-stained scaffolds. Therefore, it could be hypothesized that the bead-like globular structures attached to ECM scaffolds correspond to the stained collagen. Nevertheless, this hypothesis should be confirmed using immunohistochemistry for specific collagens.

As a measure of proliferation, the metabolic activity of cells was analyzed on days 10, 14, 17 and 21. An initial increase in metabolic activity of cells seeded on scaffolds in BM and CM was observed for the first 2 weeks of culture (figure 2). Scaffolds cultured in CM showed a higher number of cells (monitored over the period of 3 weeks) than those cultured in BM. TGF-β3 and DEX are known to promote and regulate chondrocyte proliferation and differentiation [77, 78], thereby explaining the increased cell amount in CM. Furthermore, when cells were cultured on scaffolds in the presence of ECM cues an increase in metabolic activity was also observed. Similarly to CM, the presence of ECM components may have triggered a faster cell proliferation compared to PCL fibers alone.

Chondrocytes are defined by their ability to produce characteristic ECM molecules [79] such as aggrecan [80], collagen type II [81], and sox-9 [82], and in lesser amount collagen type I [42] and collagen type X. To investigate changes in the gene expression of OA and HL chondrocytes, real time qPCR analysis was performed to measure their gene expression profile. In normal healthy cartilage, ECM is continuously remodeled. Disturbance in the remodeling process could affect the composition and function of ECM, consequently altering the properties of articular cartilage. Progressive imbalance in the expression of cartilage-specific genes aggrecan and collagen II is a typical signature of osteoarthritis initiation and progression. Cells increase collagen type I expression, while reducing the expression of aggrecan and collagen II [81, 83–85]. According to our findings, the expression of collagen X, sox-9 and collagen I was highly variable after 21 days and significantly different only in response to soluble morphogenic signals present in CM. Interestingly, HL cells seeded on hMSC ECM showed a significant increase in aggrecan and collagen II (figure 4) expression compared to HL cells cultured on hMSC ch ECM scaffolds and scaffolds without ECM. A trend was observed in the case of CM, indicating a strong influence of TGF-β3 and DEX on stem cells microenvironment [77, 78]. However, the ECM included in the scaffold failed to change the gene expression profile of OA cells. A possible explanation could be that cells sense a more *in vivo*-like milieu when surrounded by ECM secreted by undifferentiated stem cells [86, 87]. Peterson *et al* showed that dedifferentiated chondrocytes share features with primitive mesenchymal cells and high cell culturing density mimics the pre-chondrogenic cell condensation resulting in cartilage formation [88]. Bessa *et al* reported the mechanism pathway of TGF-β superfamily, where binding to serine–threonine kinase receptors on the cell surface triggers a signaling pathway that modulates the gene transcription with other transcription factors present in the surrounding ECM and regulate cell processes like

proliferation and differentiation [89, 90]. The hMSC ECM contains favorable structure, growth factors and signaling pathways, providing a microenvironment very similar to that of native ECM. The interactions between HL chondrocytes and hMSC microenvironment may contribute to induce an effect on the fate of HL chondrocytes. Thus, decellularized hMSC undifferentiated ECM is a promising candidate to impart instructive properties to 3D scaffolds. Cell-derived ECM can be fabricated by the electrospinning technique and contribute to the further development of biologically active scaffolds in cartilage tissue engineering.

Conclusion

The potential of cell-derived ECM on OA and HL chondrocytes was evaluated in this study. Our approach provides a means to mimic the *in vivo* extracellular microenvironment *in vitro*. ECM-incorporated scaffolds were fabricated using electrospinning. Decellularized ECM was obtained from human mesenchymal stem cells in basic and chondrogenic medium, resulting in different structural characteristics. Picrosirius staining confirmed the presence of collagenous ECM in PCL electrospun scaffolds containing ECM. The gene expression of HL chondrocytes cultured on hMSC ECM in CM demonstrated a significant increase in collagen II and aggrecan expression. Whilst no significant influence of hMSC ECM was observed on OA chondrocytes, hMSC ECM scaffolds in the presence of soluble chondrogenic factors provide a favorable environment influencing the fate of HL chondrocytes. Thus, cell-derived hMSC ECM scaffolds could be a promising biomaterial to fabricate scaffolds for cartilage tissue engineering.

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