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Mechanical stimulations on human bone marrow mesenchymal stem cells enhance cells differentiation in a three‐dimensional layered scaffold

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Abstract

Scaffolds laden with stem cells are a promising approach for articular cartilage repair. Investigations have shown that implantation of artificial matrices, growth factors or chondrocytes can stimulate cartilage formation; but no existing strategy apply mechanical stimulation on stratified scaffolds to mimic the cartilage environment. The purpose of this study was to adapt a spraying method for stratified cartilage engineering and to stimulate the bio-substitute. Human Mesenchymal Stem Cells from Bone Marrow (BM-MSCs) were seeded in an Alginate/Hyaluronic Acid (Alg/HA) or Alg/Hydroxyapatite (Alg/Hap) gel to direct cartilage and hypertrophic cartilage/sub-chondral bone differentiation, respectively, in different layers linked with polyelectrolyte multilayer films, in a single scaffold. Homogeneous or composite stratified scaffolds were cultured for 28 days and cell viability and differentiation were assessed. The heterogeneous scaffold was stimulated daily. The mechanical behavior of the stratified scaffolds was investigated by Plane Strain Compression tests. Results showed that the spraying

process did not affect cell viability. Moreover, cell differentiation driven by the microenvironment was increased with loading: in the layer with Alg/HA, a specific extracellular matrix of cartilage, composed of GAGs and type II collagen was observed, and in the Alg/Hap layer more collagen X was detected. Hydroxyapatite seemed to drive cells to a hypertrophic chondrocytic phenotype and increased mechanical resistance of the scaffold. In conclusion, mechanical stimulations will able to produce a stratified bio-substitute, laden with hBM-MSCs, which is capable *in vivo* to mimic all-depths of chondral defects, thanks to an efficient combination of stem cells, biomaterial compositions and mechanical loading.
Keywords: stratified tissue engineering; human mesenchymal stem cells; spraying process, hydrogels; articular cartilage; mechanical stimulations.

1. Introduction

The challenge posed for tissue engineering (TE) is to restore tissue functions by building structures like the native tissue of cells and the extracellular matrix (ECM). TE is based on three main aspects: the scaffold, the cells and the culture conditions (with or without biochemical and/or mechanical stimulations) (Stoltz et al., 2005). In this field, one of the greatest concerns relates to scaffolds which must be successfully laden with cells or colonized *in vivo* by autologous cells, and used to replace injured tissues by grafting (Mallett and Korbutt, 2009). Currently the diversity of biomaterials (e.g. natural or synthetic, hydrogels or sponges) allows for the design of scaffolds which can be used to engineer different tissues *in vitro* to mimic the native tissue function. In designing these scaffolds, certain conditions must be accomplished in order to be suitable for use in clinical applications: biodegradability, biocompatibility, no-immunogenicity and no-toxicity. For example, sodium alginate is one of the biomaterials used in tissue engineering (Wang et al., 2008) and could be supplemented with an extracellular matrix component such as hyaluronic acid and hydroxyapatite (Laydi et al., 2013).

Until 5 years ago, conventional TE provided only homogeneous scaffolds, which included one cell phenotype and an unvarying extracellular *neo*matrix (Nguyen et al., 2011). However, the majority of adult tissues have a spatially-varying matrix composition and microstructure. For example, the articular cartilage architecture is characterized by only one cell-type (chondrocytes), but with several degrees of differentiation leading to a zonal organization of the ECM, and a depth-dependent mechanical behavior, to resist stresses that vary between layers. Up to now, strategies are available, such as the Brittberg technique, but few provide satisfying results (Brittberg et al., 1994). Lesions can be solved by inducing new tissue synthesis with a stronger matrix quality, potentially containing specific cartilage cells from the onset.

For cartilage regeneration, most cells used originate from the articular cartilage. However, chondrocytes are well-known to dedifferentiate them during the monolayer amplification (Brodkin et al., 2004), and collecting chondrocytes from human healthy donors is difficult. Some studies are using primary cells or lineages of mesenchymal stem cells to avoid the weaknesses of chondrocytes, or both cells (Dahlin et al., 2014). The advantages of these cells are their ease of use, their self-renewal, their ability to obtain a large variety of phenotypes and to make autologous grafts (Pittenger et al., 1999).

To mimic multiphasic tissues, such as cartilage, the idea of stratified scaffolds was developed to obtain the original tissue structure (Thorpe et al., 2013). Until now, stratified scaffolds were prepared using different processes, such as degelling-gelling alginate hydrogel (Lee et al., 2007), by photopolymerizing gel, with electrospinning (Grey et al., 2013), ceramic construction or more recently by 3D bioprinting (Kesti et al., 2015).

In this same way, thin stratified scaffolds were built up by spraying alternate layers alginate gel and polyelectrolyte multilayers film (PEMs) article (Jessel et al., 2006) with the layer-by-layer method which can also be done by dipping (Schiavi et al., 2015). In parallel, we noticed that the spraying process has been used to restore thin tissue integrity, by spraying keratinocytes on wounded skin to accelerate healing (Grant et al., 2002), melanocytes to restore skin color (Navarro et al., 2001), or bladder urothelial cells (Hafez *et al.*, 2003).

It is well known that articular cartilage is constantly exposed to mechanical stimuli, such as dynamic and static compression, hydrostatic pressure, rotation and fluid shear (Guo et al., 2016). Mechanical loading represents an important factor, which regulates and maintains the chondrocyte phenotype and the layered design of the tissue. The motion and loading of joints are necessary for the structure, function and metabolism of native articular cartilage. *In vitro*, it is demonstrated that mechanical stimuli, such as dynamic compression, hydrostatic pressure or low-frequency vibrations, applied to human MSC-seeded scaffolds, encourage MSC to differentiate them and produce specific cartilage matrix (Li et al., 2016). A large number of studies show the beneficial effect of compression on chondrogenesis, resulting in a significant increase in cartilage-specific extracellular matrix genes and proteins expression such as type II collagen and aggrecan (Zhang et al., 2016). *In vitro* compression systems are controlled and allow to simulate the *in situ* stress conditions (Guo et al., 2016).

In this work, the layer-by-layer process has been used to spray a stratified scaffold, based on alginate and laden with BM-MSC, aimed at being mechanically stimulated to mimic cartilage zonal organization and environment. To successfully obtain an artificial construct with cartilage specificity, we attempted to construct layered scaffolds by spraying alternate layers of gel, and

polyelectrolytes film (PEM) which linked the hydrogel layers together with electrostatic interactions. Two scaffolds were built: one with two similar layers, to assess the ability of maintaining the bio-substitute in culture for 4 weeks, and the second, a bio-substitute with two different layers (composite), to mimic the microenvironment of cells in cartilage tissue. In this way, alginate gel was supplemented with cartilage or bone matrix components (hyaluronic acid (HA) or hydroxyapatite (Hap), respectively), and then seeded with human mesenchymal stem cells from bone marrow (BM-MSCs) before the scaffold construction. Before spraying the second layer, Poly-L-Lysine and Hyaluronic Acid (PLL/HA) were sprayed on the hydrogels to obtain PEM. Then, daily compression was applied for 4 weeks on the bio-substitutes and were compared to those that remained without loading. For 28 days, the capacity of matrix components which target BM-MSCs differentiation (in combination with loading) into chondrogenic or osteogenic phenotype was assessed. In parallel, biomechanical behavior of the scaffolds was studied.

2. Materials and methods

2. 1. Bone Marrow human Mesenchymal stem cells culture

Mesenchymal stem cells from human bone marrow were collected from marrow aspirate of donors, taken during total knee replacement (age 50-70 years, 5 donors). Bone marrow was collected after patients' informed consent; this complied with national legislation regarding human sample collection, manipulation and personal data protection. These biological samples were regarded as surgical waste and therefore, following the opinion of an ethics committee of Nancy Hospital, no authorization of this committee was necessary for the sample collection. Cells were counted and cultivated up to the third passage, as previously described (Tritz-Schiavi et al., 2010). At the end of their expansion, cells were removed from the support and embedded in an alginate gel to create the stratified scaffold.

2. 2. Spraying of the stratified scaffold laden with BM-MSCs

The stratified scaffold was built with two layers composed of 1.5% Alginate/Hyaluronic Acid (w/v with 80% of Alg and 20% of HA diluted in 0.9% of NaCl) or 1.5% Alginate/Hydroxyapatite microparticles (w/v ratio 95:5, Alg/Hap) and laden with BM-MSCs. Layers were welded by 5.5 PEMs bilayers of Poly-L-Lysine/HA (PLL/HA) (Fig. 1). The spraying method was previously used by our group with rat chondrocytes (Tritz et al., 2010), and BM-MSCs (Tritz-Schiavi et al., 2010). Two different scaffolds were constructed by spraying varied gel volumes to differentiate them: one with two layers of Alg/HA (named

Alg/HA 2X, 6 mL for the deep layer, and 4 mL for the upper one), a composite scaffold with one layer of Alg/Hap and the upper one with Alg/HA (Alg/HA-Alg/Hap, same ratio).

In brief, BM-MSCs were seeded at $3x10^6$ cells mL^{-1} in 1.5% Alg/HA or Alg/Hap gel, then the 6 mL of cells suspension was sprayed at 0.9 bar on a sterile glass plate with an airbrush connected to a compressor. The glass plate was dipped into a $CaCl_2$ bath at +37°C (102 mM) and gelation was carried out for 10 min. Finally, the hydrogel was washed twice with a washing solution (NaCl at 0.9%, 10 mM Tris, pH 7.4). To weld this hydrogel layer with the top layer, PEMs were sprayed on it. A polycation (PLL at 1 mg/mL in washing solution) was sprayed for 5 sec and washed twice for 10 sec with the washing solution, then, a polyanion (HA at 1 mg/mL in washing solution) was sprayed and washed, following the same protocol. PEMs were built up with 5.5 bilayers of PLL/HA. Finally, the second layer was built up using the same method as the first layer with less gel volume (4 mL). Scaffolds were cultured for up to 28 days, with a chondrogenic medium and without any growth factor: DMEM-high glucose (Gibco, Grand Island, NY) supplemented with 10 % fetal bovine serum (Gibco), 2 mM Glutamine (Sigma), 100U/mL/100 µg/mL Penicillin/Streptomycin (Sigma), 2.5 µg/mL Amphotericin B (Sigma), 100 µg/mL Sodium Pyruvate (Sigma), 40 µg/mL L-Proline (Sigma), 50 µg/mL L-Acid Ascorbic (Sigma), 100 nM Dexamethasone (Sigma) and 1 mM $CaCl_2$ (Sigma) (Tritz-Schiavi et al., 2010). For mechanical testing, scaffolds were made without cells.

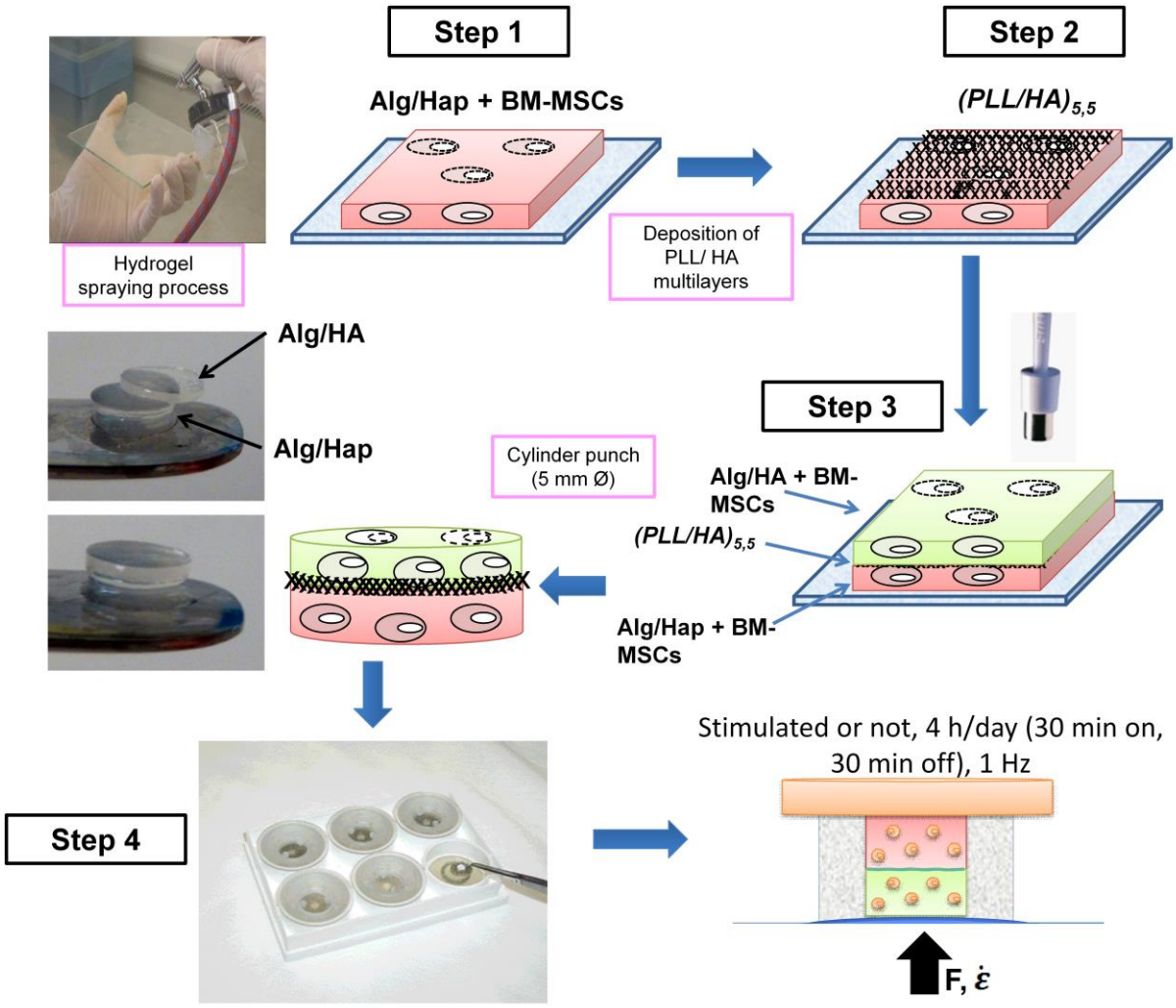

Figure 1. Preparation of stratified scaffold. The proposed way is highly innovative and consists in a simple and progressive spraying of all the elements constituting finally the layered biomaterial. The build-up strategy is principally based on the alternate deposition by spraying of: (i) cells (human mesenchymal stem cells from bone marrow, BM-MSCs) embedded into alginate gels supplemented with HA or Hap, then gelation (Alg/HA or Alg/Hap, **Step 1**), (ii) Poly-L-Lysine (PLL)/ hyaluronic acid (HA) multilayers (5.5 layers, **Step 2**), (iii) BM-MSCs embedded into Alg/HA hydrogel then gelation, and samples are cut with a biopsy punch (**Step 3**). Multilayered polyelectrolyte film $(PLL/HA)_{5.5}$ links hydrogels thanks to electrostatic interactions. Finally, half of bio-substitutes are submitted to a daily cyclic strain (**Step 4**).

2. 3. BM-MSCs tracking by confocal microscopy

BM-MSCs were tagged with viable fluorescent labeling: PKH 26 (Sigma) interacts with the phospholipids of plasmatic membranes and is excited at $\lambda = 551$ nm, and Syto 16 (Molecular Probes™) labels nucleic acid and is excited at $\lambda = 488$ nm. Cells were detached from the flask, centrifuged, washed twice with PBS and stained with PKH 26 or Syto 16 (10 µM) in 1 mL of final solution during 5 and 15 min, respectively. Then, stained cells were homogenized with complete medium and washed twice. Cells were counted and could be used for scaffold construction. During the *in vitro* culture, scaffolds were kept in the dark to avoid fluorescence

bleaching.

After 3 and 7 days of culture, confocal microscopy allowed for tracking cell migration. For all labeling, fluorescence emission was detected using Confocal Laser Scanning Microscopy (CLSM, Leica TCS SP2 equipped with an acousto-optical beamsplitter, Confocal Microscopy Laboratory-PITC of FR-CNRS 3209). Excitation was achieved by the 488 nm line from an Ar laser for Syto 16 labeling, and the 543 nm line from a He/Ne laser for PKH 26. All photomultipliers used were adjusted to the same sensitivity (gain/offset).

2.3 Mechanical stimulations

From day 3, half of the cell laden scaffolds were subjected to mechanical stimulation, until day 28. In our study, intermittent dynamic compression was applied to scaffolds at 220 kPa, 1 Hz, for 30 min of loading and 30 min of unloading for 4h/day. These settings were chosen because they showed a better response from stem cells in Alg/HA monolayer scaffolds, in a previous study (data not published). This mechanical stress was performed using a compression system Flexer cell FX-4000™ (Flexcell International, US) which was a patented, computerized, pressure-operated instrument that applied a defined controlled compression. Pressure signal and frequency settings were controlled by Flex Soft™ 4.0 software (Flexcell International, US). Stratified scaffolds were plated up-side down in six-wells compression plates, to allow the compression to be applied on the Alg/HA layer and were compressed between a piston and a stationary platen. The piston was attached to the flexible-bottomed rubber membrane and was moved upward by positive air pressure applied to this membrane. Basic chondrogenic medium was used and changed twice a week.

2. 4. Cells viability analysis by flow cytometry

Apoptotic and necrotic cells were analyzed by flow cytometry using the Vybrant/Apoptosis™ kit, based on the Annexin V/Propidium Iodide staining procedure (Molecular Probes, France). Briefly, cells were taken off of the Alg/HA hydrogel by dissolution in 55 mM sodium citrate and 50 mM EDTA solution, after centrifugation (320*g*, 5 min), cells were suspended in 100 µL of Annexin-liant buffer with 2.5 µL of Annexin V-Alexa 488 and 1 µL of Propidium Iodide (PI). After 15 min of incubation, 200 µL of Annexin V buffer was added to each sample. Then, cells were analyzed by measuring fluorescent emission at 530 nm and 575 nm, respectively for Alexa 488 and PI with flow cytometer (BD FACS flow cytometer). For all analyses, at least 5 000 events were taken in account.

2. 5. Gene expression analysis by quantitative real-time PCR (qRT PCR)

Cells were extracted from the hydrogel by chelating Ca^{2+} with citrate-EDTA and were washed 3 times with PBS. First, RNA extraction was completed with the RNeasy extraction kit (Qiagen) and purified with a DNase kit (Qiagen). Then, cDNA was obtained by the reverse-transcription of mRNA with the iscript kit (Biorad). Finally, gene expression was studied by doing a qRT PCR with the QuantiTect SYBR Green PCR kit (Qiagen) specific primer (Table 1) and cDNA obtained from samples, on a Light Cycler system (Roche Diagnostics) for 45 cycles to perform a quantitative analysis of the gene expression. All gene expression was reported to the housekeeping gene RP-29.

Table 1. Sequences of primers used in quantitative real-time polymerase chain reaction.

Gene name	**Primer sequence (5' – 3')**	**Tm**
Coll2 total	Reverse GAACCTGCTATTGCCCTCTG Forward ATGACAATCTGGCTCCCAAC	55°C
Agg	Reverse TCGAGGGTGTAGCGTGTAGAGA Forward TCGAGGACAGCAAGGCC	62°C
COMP	Reverse TCTGCATCAAAGTCGTCCTG Forward ACAATGACGGAGTCCCTGAC	60°C
Sox9	Reverse CCTGGGATTGCCCCGA Forward GAGCAGACGCACATCTC	55°C
Coll10	Reverse CTCCAGGATCACCTTTTGGA Forward GCTAAGGGTGAAAGGGGTTC	60°C
Runx2	Reverse CGTTACCCGCCATGACAGTA Forward CCCGTGGCCTTCAAGGT	60°C
RP29	Reverse AGACGCGGCAAGAGCGAGAA Forward AAGATGGGTCACCAGCAGCTCTACTG	60°C

2. 6. Observation of extracellular matrix and scaffold structure with histology

After 28 days of culture, scaffolds were incubated in a fixation solution (4% paraformaldehyde, 100 mM sodium cacodylate, 10 mM $CaCl_2$, pH = 7.4) for 4 hours at room temperature and then left overnight in a histological washing solution at +4°C (100 mM sodium cacodylate, 50 mM $BaCl_2$, pH = 7.4). Then, they were dehydrated through a series of increasing grades of ethanol,

cleared with toluene and embedded in paraffin wax. Sections were cut at a thickness of 5 µm using a rotary microtome and mounted on glass slides. For histological investigation, the slices were stained using Sirius Red (SR) or Alcian Blue at pH = 1 (AB) staining for matrix content observation. The observation in brightfield light microscopy showed matrix synthesis by cells into the scaffold: SR the total collagen, and AB the proteoglycans.

2. 7. Repartition of collagens by confocal microcopy

After 28 days, samples were fixed as for histology process and type II and X collagens indirect immunofluorescence labeling were done. Then, samples were washed three times with PBS, and then blocked with 0.5% bovine serum albumin (BSA) in DMEM without phenol red (blocking solution) for 15 min. Specimens were incubated for 45 min with rabbit anti-human type II or X collagen polyclonal antibodies (Calbiochem, France). After washing with blocking solution, samples were incubated (45 min) in the dark with secondary antibodies: a goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes). After two washes, cylinders were observed. For all labeling, Alexa Fluor 488 fluorescence emission was detected using a confocal laser scanning microscopy (CLSM) and observed as mentioned previously for Syto 16.

2. 8. Mechanical behavior of scaffolds

In this study, the scaffolds were built up in the form of thin sheets, which makes the use of traditional mechanical tests inadequate. For more details on the rheological models developed specifically for alginate, one may refer to the review by Moresi et al. (considering relaxation, creep and complex properties in dynamic straining) (Moresi et al., 2001), by Nussinovitch et al. (using modified Maxwell model) (Nussinovitch et al., 1989), and by Magnenet et al. (Magnenet et al., 2012).

The Plane Strain Compression test, (PSC test), or the Watts-Ford test, usually used in metal forming and tribological simulation, was performed on those alginate based scaffolds as described previously (Tritz et al., 2010, Magnenet et al., 2012). From a classical modeling of the plastic deformation in the PSC test, it is also reported (Montmitonnet et al., 2000), that the equivalent Von Mises stress, $\bar{\sigma}$, and strain, $\bar{\varepsilon}$, may be written as

$$\bar{\sigma} = \left[\frac{\frac{\sqrt{3}}{2}}{1 + \bar{m}\frac{a}{4(h_0 - \delta(t))}}\right]\frac{F(t)}{2aL_0}$$

$$\bar{\varepsilon} = \frac{2}{\sqrt{3}}\ln\left(\frac{h_0}{h_0 - \delta(t)}\right)$$

where h_0, L_0, $\bar{m}$, are the initial thickness, the initial length and the friction factor, respectively. All the PSC tests were carried out in a bath of 0.9% NaCl solution at human thermal condition (+37°C), and with negligible friction effect (estimated at $\bar{m}$ = 0.05).

The experimental stress-strain responses were obtained from 24 specimens (3 for each condition and per strain-rate), it was performed at the same time to minimize the effect of preparation methods as alginate sterilization, and of the strain-rate sensitivity.

2. 9. Statistical analysis

All data are presented as means ± standard error means (SEM) for a minimum of 3 independent experiments, which contain 3 biological repeats each, except for the mechanical behavior results. A second way ANOVA was used to determine if significant differences existed for the mixed-population experiment. A *p*-value less than 0.05 meant a significant difference for the ANOVAs. If significance existed, a post-hoc analysis was performed using the Tukey's multiple comparison tests for all experiments.

3. Results

3. 1. Design of a stratified scaffold

3.1.1. Cells behavior in a stratified scaffold

To evaluate the ability to seed different cell types between layers, cell migration was observed during the first 7 days of culture in a homogeneous stratified hydrogel composed of Alg/HA in both layer (Alg/HA 2X). BM-MSCs were tracked thanks to a tagging with two forms of viable fluorescent labeling: PKH 26, a plasma membrane stain, and Syto 16, a nucleic acid stain (Fig. 2-A). The superficial zone, fluorescing in red, can be discerned from the deep zone, fluorescing in green. The results show us a homogeneous repartition of the cells in every layer of the scaffold and no-migration of cells with red fluorescence (PKH 26) into the layer containing cells labeled with Syto 16 (green) and conversely. In addition, to validate cell spraying, BM-MSCs viability was followed by flow cytometry during the experiment (Fig. 2-B). The proportion of living cells in the hydrogels is significantly increased between 3 and 14 days of *in vitro* culture of Alg/HA 2X hydrogels laden with BM-MSCs.

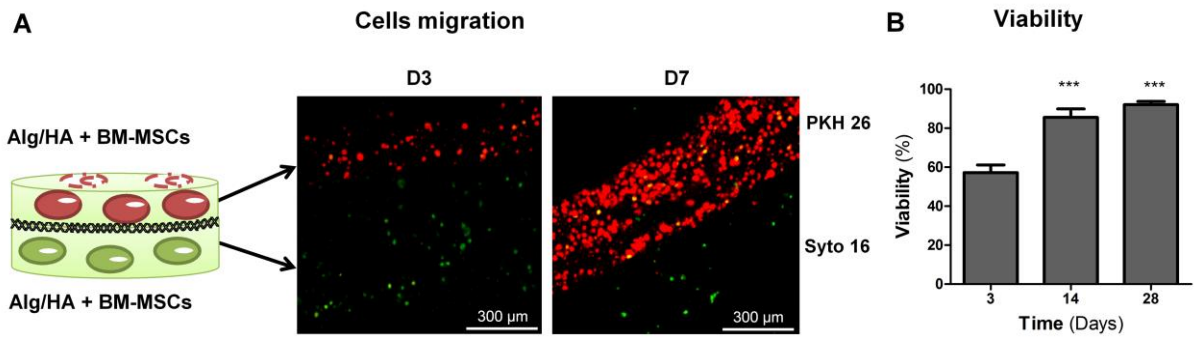

Figure 2. Cells behavior after seeding in a homogenous stratified scaffold composed of Alg/HA hydrogel. After culture, cells were divided in two groups. In the first group, nuclei of BM-MSCs were labeled with Syto 16 (green) whereas plasma membranes of cells, belonging to the second population, were labeled with PKH 26 (red). Then, each population was seeded in a distinct Alginate/Hyaluronic Acid gel (Alg/HA) before scaffold construction. Observation by confocal microscopy was realized after 3 (D3) and 7 (D7) days of *in vitro* culture (**A**). There is no cell migration up to 7 days. Scale bars represent 300 µm. After 3, 14 and 28 days, cells viability was calculated after apoptosis and necrosis labeling of cells and flow cytometry analysis of samples (**B**). A significant increase is observed after 14 days of culture. Results were represented in means ± SEM, D3 vs Dx ***p<0.001.

3.1.2. BM-MSC chondrogenic differentiation in a homogenous stratified scaffold

In order to study the ability to assess stem cell differentiation after the spraying, the Alg/HA 2X scaffold was designed and cultured up to 28 days to check chondrogenic parameters.

Gene expression of Sox9, Agg and COMP are significatively increased after 28 days of *in vitro* culture in BM-MSCs seeded in the stratified scaffold and cultured with a chondrogenic media (Fig. 3-A). Moreover, GAG and total collagen stainings show a spherical morphology in the construct and a pericellular synthesis of those two components around cells (Fig.3-B). The same cell behavior is observed in a trilayered scaffold (Supp. Fig. 1) In addition, type II and type X collagens are synthesized around cells in all layers.

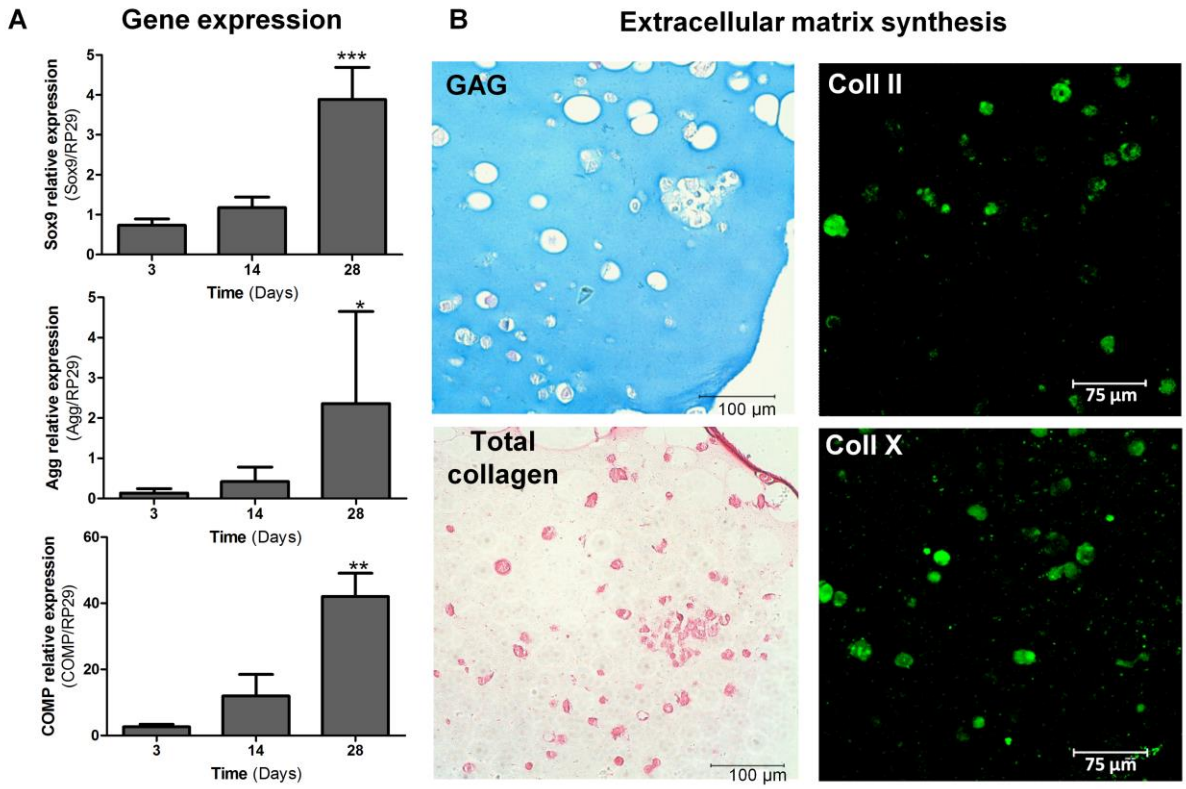

Figure 3. BM-MSCs differentiation in a sprayed homogeneous stratified scaffold. Gene expressions (**A**) of Sox9, Agg and COMP in BM-MSC seeded in an Alg/HA 2X were analyzed with qRT-PCR. All those gene expressions are increased during time in stem cells seeded in this scaffold. Results were represented in means ± SEM, D3 vx Dx * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Then, ECM synthesis was checked with histological staining with Alcian Blue for GAG and Alizarin Red for total collagens, and with immunofluorescent labeling of type II and X collagens (**B**). Cartilage markers are present: GAG, type II (Coll II) and X collagen (Coll X). Scale bars represent 100 µm on histological images, and 75 µm on fluorescent images.

3. 2. Mechanical behavior of the two scaffolds designed

To analyze the influence of biomaterials and the stratified shape on the scaffold mechanical behavior, PSC tests were provided on four types of samples: two homogeneous hydrogels composed of one layer of Alg/HA or of Alg/Hap, and two stratified scaffolds, a homogeneous hydrogel composed with two layers of Alg/HA (Alg/HA 2X), and a composite hydrogel with an upper Alg/HA layer on an Alg/Hap layer (Alg/HA- Alg/Hap) (Fig. 4). The mechanical tests were conducted at two strain rates. It has been found that the homogeneous samples Alg/HA and Alg/Hap exhibited a similar behavior at 2% s^{-1} (Fig. 4-B). However, the homogeneous samples of Alg/Hap are significantly stronger than the Alg/HA ones at 0.7% s^{-1} (Fig. 4-A). In parallel, the scaffolds Alg/HA 2X and Alg/HA-Alg/Hap show a 2.73 and 1.37 increase of their equivalent stress compared to the one-layer hydrogels Alg/HA and Alg/Hap, respectively. This phenomenon is observed for the two strain-rates. Specifically, this observation holds for strains greater than 70% at a strain rate of 0.7% s^{-1} ($p<0.05$) and 80% at 2%s^{-1} ($p<0.05$) for hydrogels

containing Hap. In addition, Alg/HA 2X had a stronger behavior than hydrogels with one layer at a strain greater than 40% for 0.7% s^{-1} (p<0.05) and at 100% at 2%s^{-1} (p<0.01).

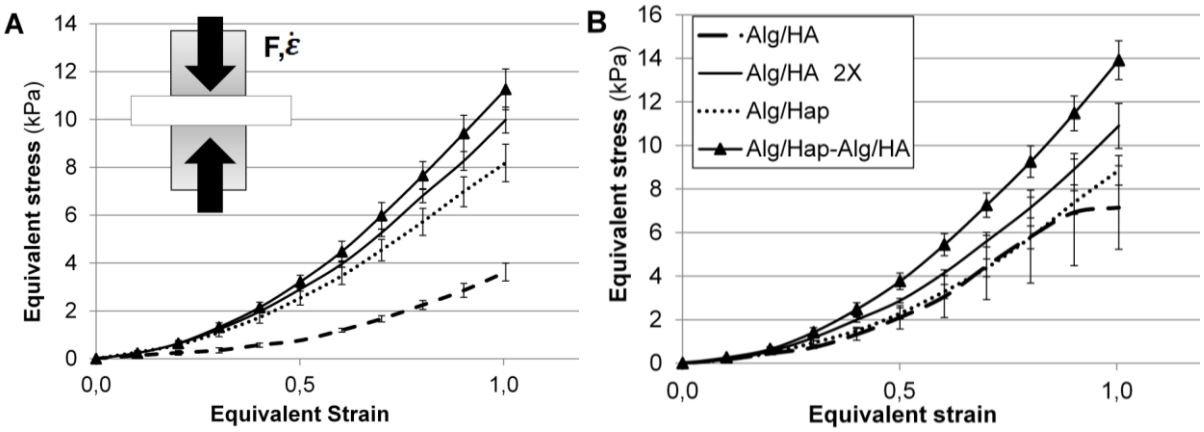

Figure 4. Influence of biomaterial composition and scaffold pattern on mechanical behavior. PSC tests were performed on scaffolds with one (discontinuous lines) or two layers (continuous lines) at two strain rates: 0.7 (**A**) or 2% s^{-1} (**B**). Equivalent stress-strain curves of Alg/Hap hydrogels with one layer were stronger than the one with Alg/HA at a strain rate of 0.7% s^{-1}. In parallel, whatever the strain rates and biomaterial composition, stratified hydrogels (Alg/HA 2X or Alg/Hap-Alg/HA) had stronger equivalent stress-strain curves than hydrogels with one layer. Results were represented in means ± SEM.

3. 3. Combination effect of BM-MSC, biomaterials and mechanical loading on chondrogenic differentiation

3.3.1 Biomaterial effects

BM-MSCs were seeded in a stratified scaffold with an Alg/Hap layer (Alg/Hap) and an upper Alg/HA layer (Alg/HA). For qRT-PCR (Fig 5-A), layers were dissociated thanks to difference in thickness (ref 2.2). Without mechanical stimulations, few statistical differences were shown. During this time, in all biomaterials, there is an increase of the absolute gene expression of ECM marker up to 28 days: COMP (p<0.01 after 14 and 28 days), Agg (p<0.01 after 28 days), Col2 and Col10 (Fig. 5-A). In addition, type II and X collagen immunostainings show similar presence in both layers after 28 days without loading (Fig. 5-B).

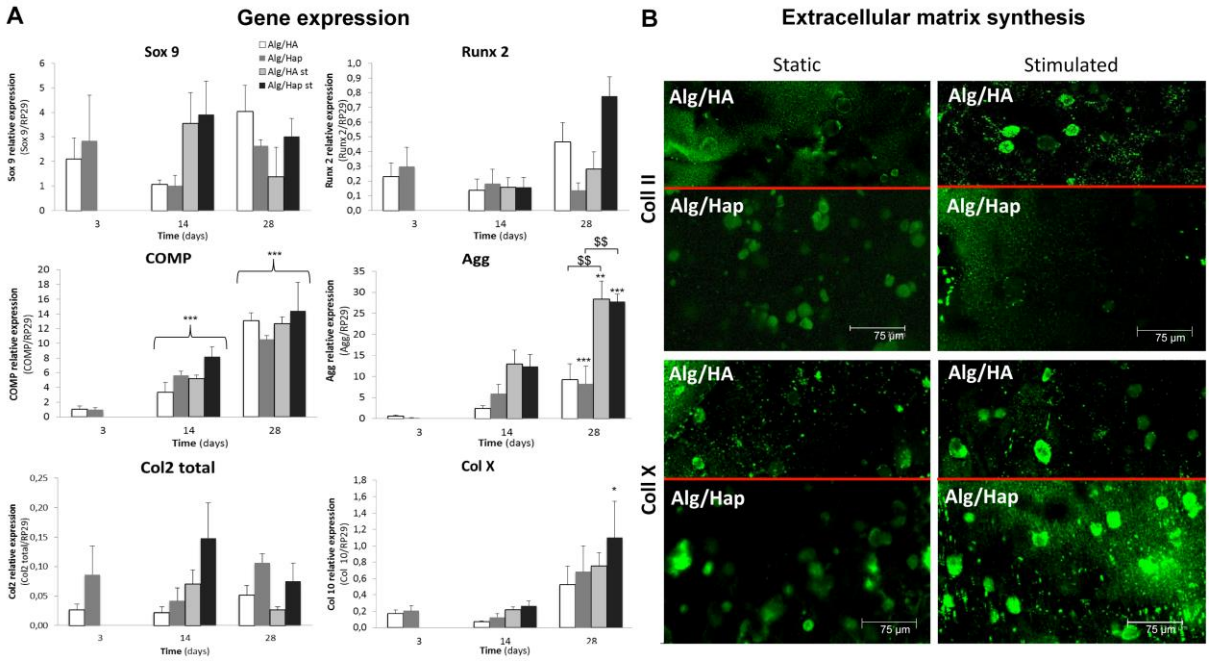

Figure 5. Influence of daily mechanical stimuli on BM-MSC differentiation. Gene expressions (**A**) of Sox9, Runx2, COMP, Agg, Col2 total and Col10 in BM-MSC seeded in an Alg/HA or Alg/Hap layers of the stratified scaffold were analyzed with qRT-PCR. COMP, Agg and Col X expressions are increased during time in stem cells seeded in all conditions: without or with mechanical stimulations (**st**). Results were represented in means ± SEM, D3 vx Dx * p<0.05, ** p<0.01, *** p<0.001 or without *vs* with mechanical stimulations $$ p<0.01. After 28 days of culture without or with mechanical stimulations, ECM synthesis was checked with immunofluorescent labeling of type II (Coll II) and X (Coll X) collagens (**B**). Cartilage marker (Coll II) is more present with mechanical stimuli stimulated in the Alg/HA layer, and hypertrophic marker (Coll X) in the layer Al/Hap stimulated. Scale bars represent 75 µm.

3.2. 2. Mechanical stimulations effect

The daily intermittent compressions on the stratified scaffold increased chondrogenic marker expression Sox 9 and Col2 total at D14, and Agg after 14 days (Fig. 5-A, at D28 p<0.01 in Alg/HA, p<0.001 in Alg/Hap layers compared to the same layer in static), and synthesis (Fig. 5-B). Type II collagen staining (Coll II) is more present in the layer Alg/HA in the stimulated scaffold, and weaker in the layer Alg/Hap. In parallel, loading increased hypertrophic chondrocyte marker expression, such as Runx2 and Col 10 (p<0.05) at D28 (Fig. 5-A) and type X collagen synthesis (Fig. 5-B), but this only occurred in the Alg/Hap layer.

4. Discussion

In this study, we establish for the first time, to our knowledge, that a heterogeneous stratified scaffold seeded with primary human BM-MSCs can be loaded daily for 28 days and that it increases the effect of the biomaterial composition. First, we validated the process with a stratified scaffold, which contained only alginate supplemented with hyaluronic acid in the two layers, by showing a good viability of cells, and chondrogenesis stem cells laden in the

hydrogel. In parallel, we designed a stratified scaffold that allows stem cells to differentiate in a depth dependent manner and to mimic all cartilage layers. Indeed, we observed specific gene expressions and protein syntheses in the function of the biomaterial composition and the effect is increased when loads are applied. When daily stimulations are applied, an increased effect of biomaterial composition on the cell differentiation was observed: type II collagen which is specific to articular cartilage, is more synthesized in the Alg/HA layer, and type X collagen is more present in the Alg/Hap layer. On this specific point, Nguyen *et al.* showed that varying composition of biomaterials supplemented with compounds of the cartilaginous matrix, seems to target BM-MSCs differentiation in different ways (Nguyen et al., 2011). Moreover, composition and mechanical behavior of biomaterials seem to play an important role in the cells differentiation, and as a matter of fact, on the ECM synthesis (Lutolf et al., 2009, Nguyen et al., 2011).

Plane strain compressive (PSC) tests were done at different strain rates to compare compound effect and stratification influences on mechanical behavior of hydrogels. Strain rates were chosen based on our previous study which showed an increasing equivalent stress in alginate hydrogels when the strain rate increased up to 2.5% s^{-1} (Tritz et al., 2010). Moreover, a strain rate at 2% s^{-1} was used because it fits to the mean strain (2.8%) of the articular cartilage after a 5 min walking, for which frequency is commonly appreciate at 0.33 Hz (Eckstein et al., 2005). Here, PSC tests showed that mechanical behavior of hydrogels was stronger when hydroxyapatite was added and softer with hyaluronic acid at a 0.7% s^{-1} strain rate. Those results are similar to the work of Lin *et al.* that showed an increasing mechanical resistance and elasticity with the improving of hydroxyapatite quantity in a PLA (Poly Lactic Acid) scaffold (Lin et al., 2007). In parallel homogeneous stratified scaffolds (composed only with Alg/HA and polyelectrolytes) have a stronger resistance than the Alg/HA hydrogel with one layer. In the same way, composite stratified hydrogels had a significantly stronger behavior than hydrogel containing only Hap or HA and with only one layer. Therefore, PEMs seem to play an important part in the strain resistance. Mechanical test as AFM (Atomic Force Microscopy) were performed to explore influences of the height and the concentration of PEMs (Boudou et al., 2009). Indeed, Boudou *et al.* showed that Young modulus increases linearly with the number of PEMs bilayers of PLL/HA. Yet, any work until now had shown an increase of mechanical behavior of hydrogel scaffold containing PEMs with this build-up process. The use of PEMs is well known to induce cell adhesion or to deliver active molecules (Mendoza-Palomares et al., 2012). However, the results of this study showed that use of PEMs allow for long term cohesion of a stratified scaffold, an increase of scaffold mechanical properties and

the fabrication of bi-stratified and tri-stratified scaffolds which remained constant up to 56 days of *in vitro* culture (data not shown).

In this study, the cell migration was examined at the interface of layers using a confocal microscope to show the ability of the process to keep cells on the same layer. After 7 days, we had seen no migration of fluorescent stained cells across these interfaces. Thus, we can assume that not only can the PEMs weld the hydrogel layers, but it also prevents cell migration from one layer to another. Moreover, the use of PLL in the PEMs seems to be judicious for cartilage engineering if we take in consideration the results from the work of Woodward *et al.* In fact, mesenchymal cells of embryonic limb bud were driven in a chondrogenic manner when they were cultured in the presence of PLL (Woodward and Tuan, 1999).

In previous studies, we have shown and explained that the spraying method had few effects on cell viability and metabolic activity at the onset of culture (Tritz et al., 2010, Tritz-Schiavi et al., 2010). In this work, the spraying method seems to not be deleterious for BM-MSCs as seen from their good viability and their ability to differentiate into a chondrogenic phenotype in the presence of HA or to a hypertrophic phenotype with Hap. Indeed, the results indicate increasing of Col10 and Runx2 expression in stimulated scaffolds containing Hap which are specific to hypertrophic chondrocytes. The influence of Hap is observed in the static condition for Col10 expression in the Alg/HA layer of the composite scaffold, which did not contain this Hap, whereas in the Alg/HA 2X scaffold this increase is not observed (Supp. Fig. 2). At this time, it is well known that biomaterial composition/properties can direct cell differentiation, then ECM synthesis (Lutolf et al., 2009, Nguyen et al., 2011), and moreover, that cells may influence each other in a co-culture system. In fact, Hap is commonly used in osteochondral engineering, and is recognized for directing cell fate to an osteogenic phenotype (Oliveira et al., 2006).

In parallel, Chung *et al.* had demonstrated that HA-based scaffolds support BM-MSCs chondrogenesis thanks to the potential for cell-scaffold interactions *via* cell surface receptors (Chung and Burdick, 2009). As we showed in this work, a chondrocytic ECM is synthesized as a pericellular type II collagen and GAGs. In this way, it indicates that HA biomaterial provides a beneficial niche for BM-MSCs chondrogenesis. Thus, those biological results indicate that we have a variable differentiation in this heterogeneous scaffold and that mechanical stimuli increase this effect.

Moreover, articular cartilage is a thick tissue with a height between 1 and 1.62 mm in the ankle and 1.69 and 2.55 mm in the knee (Shepherd and Seedhom, 1999). Here, the stratified scaffolds provided had a mean height of 1.66 mm for homogeneous bistratified scaffolds, 1.7 mm for composite scaffolds and 1.69 mm for tri-stratified scaffolds for the same total gel

volume (data not shown). Thus, this process allows for filling of deep chondral or osteochondral defects in the ankle and knee by increasing the volume of gel sprayed. Finally, this process provides a bio-substitute that is ready for implantation and can allow stem cells to differentiate directly in the defect, where the daily strain will increase cell differentiation in a biomaterial dependent pattern.

Conclusions

The present study demonstrates that this smart combination of human stem cells, biomaterial composition and mechanical loading in a stratified scaffold can provide a high-quality bio-substitute for cartilage defects. Indeed, stratified scaffolds can be built with alginate gel, laden with primary human mesenchymal stem cells from bone marrow, which can fill deep cartilage or osteochondral defects. After 8 weeks of *in vitro* culture, not only was the specific extracellular matrix synthesized in HA and Hap biomaterials, but the layers were also still welded. All these results are encouraging for the next step, which is to perform *in vivo* grafting of these stratified scaffolds because of the ability of loading to increase the microenvironment's influence on stem cell differentiation. Moreover, polyelectrolyte multilayers have a great relevance in this innovative technology. They not only allow cohesion between Alginate/cell layers but will also serve, if needed, as bioactive reservoir for growth factor or other drugs, to locally induce a specific cell differentiation. Furthermore, different combinations of cell types and biomaterials can be tested to obtain a *neo*cartilage with a spatially-varying cell phenotype and extracellular matrix. This method can be applied for other stratified tissues, such as blood vessel or skin. Finally, the good quality cohesion and mechanical behavior of this stratified scaffold makes it suitable for transplantation *in vivo* into deep cartilage or osteochondral defects, and *in vivo* mechanical stimulation induces a specific ECM synthesis.

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Author Contributions statement

JS, contributed to conception, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; LR, contributed to data acquisition, analysis, draft and critically revised the manuscript; NC, contributed to data acquisition and analysis; NdI, NBJ, JFS contributed to conception, interpretation, critically revised the manuscript; DM contributed to conception, cells collection from the hospital, and critically revised the manuscript; RR and CH supervised the project and contributed to conception, analysis, drafted and critically revised the manuscript.

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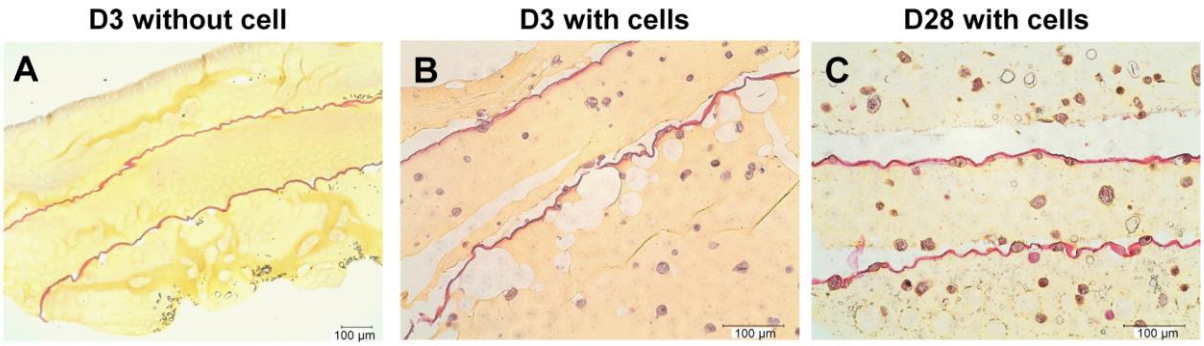
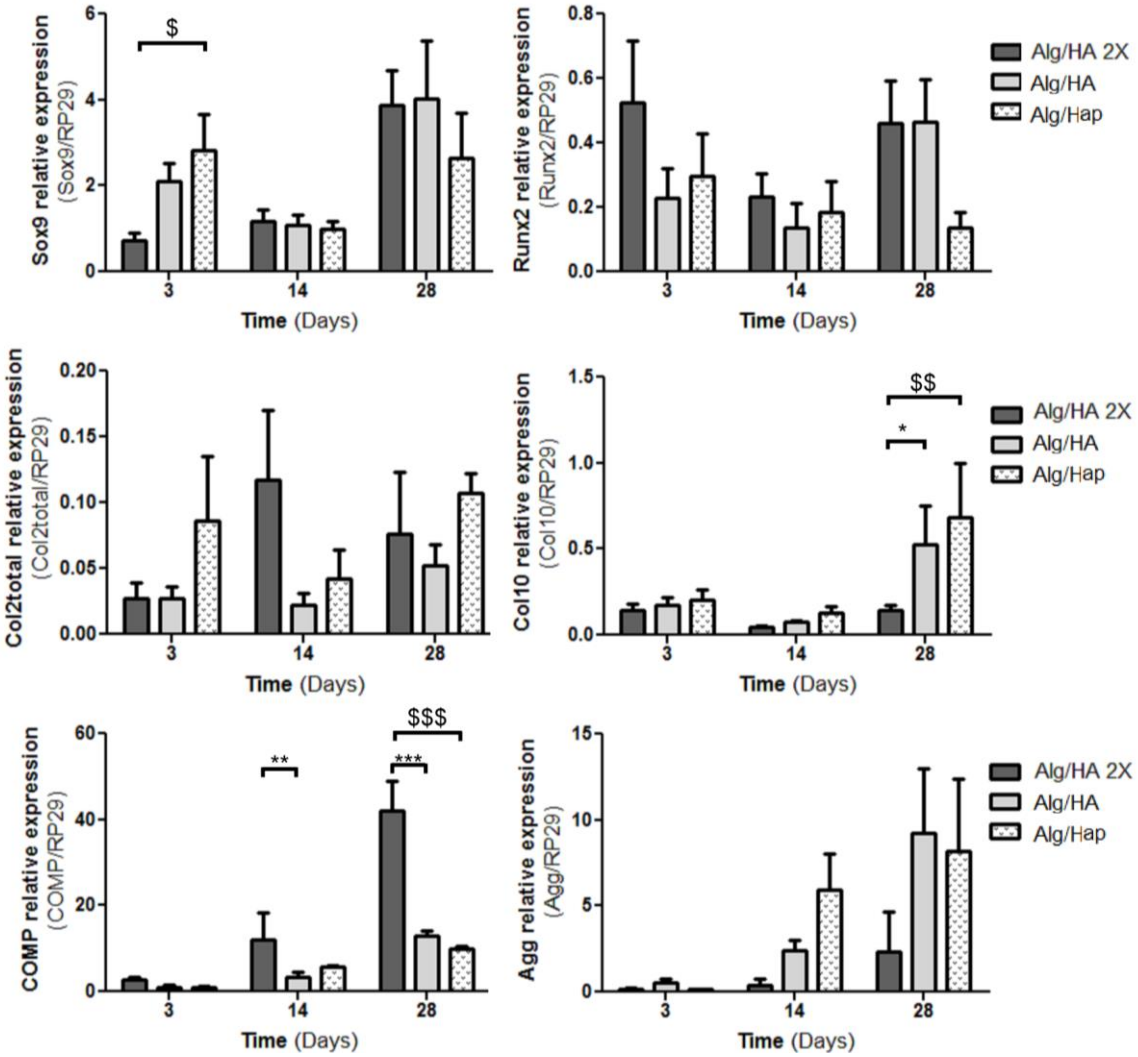


Supplemented Figure 1. Stratified scaffolds were built up with three layers of Alg/HA without (**A**) or laden with cells (**B - C**). Scaffolds were stained with HES after 3 days of culture (**A - B**) or 28 days of culture (**C**) and observed at 10X objective (A) or 20X objective (B - C). Cohesion of all the 3 layers is show up to 28 days of culture. Scale bars represent 100 µm.



Supplementary Figure 2. Influence of biomaterial composition on genes expression without mechanical stimulation. Real Time PCR (average of relative expression) showed that biomaterial composed with only Alg/HA showed an earlier type II collagen gene (Col2 total) expression, and a significant and stronger COMP expression after 28 days of culture. The presence of Hap in the scaffold induced a significantly increase of type X collagen gene relative expression (Col10) compared to condition of stratified biomaterials composed only with Alg/HA. Whatever the biomaterial and scaffold, Sox9 and aggrecan (Agg) genes expressions increased and Runx2 gene expression was regular during the time of culture. Results were represented in means ± SEM, Alg/HA 2X *vs* Alg/Hap $ p<0.05, $$ p<0.01, $$$ p<0.001, Alg/HA 2X *vs* Alg/HA * p<0.05, ** p<0.01, *** p<0.001.