Exploring the effects of the growth factor BMP-9 on articular cartilage-derived progenitor cells to enhance articular cartilage regeneration

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INDEX

ABSTRACT

Articular cartilage lacks intrinsic repairing potential, thus, osteochondral lesions might develop degenerative and lasting defects. These defects lead to pain and disability in young adults, and when these are left untreated, they can develop into early Osteoarthritis (OA). To overcome this problem, cell therapies offer healing and functional replacement of the damaged tissue. In the regenerative medicine field, chondrocytes and Mesenchymal Stem Cells (MSCs) have previously been studied to regenerate the articular cartilage. Recently however, Articular Cartilage Progenitor Cells (ACPCs), a sub-population of chondrocytes, have emerged as an alternative to use in these therapies. Choosing the medium conditions and the correct growth factors, in order to enhance the chondrogenic potential of these cells, has been a major challenge within this field. In contrast with previous studies which used growth factors such as Transforming Growth Factor Beta-1 (TGFβ1) and different Bone Morphogenic Proteins (BMPs), this project focused on exploring the chondrogenic potential effects of BMP9 on ACPCs. Because other factors, such as cell environment and scaffold, are also crucial to improve the cell differentiation and integration in the cartilage native tissue, different three-dimensional (3D) structures were also studied under BMP9 conditions. Norbornene Acid Hyaluronic modified hydrogels (NorHa) were used to improve the chondrogenic differentiation of ACPCs, and polycaprolactone (PCL) 3D printed meshes were assessed as possible articular cartilage implants to guide and reinforce the chondrogenic differentiation.

LAYMAN'S SUMMARY

Articular cartilage is the connective tissue that covers the end of the long bones of our body, such as knees or shoulders. This tissue has a low healing potential, thus, after successive impact, an accident or any other lesion this can suffer serious and long-lasting damage. Over the last few years, regenerative medicine has evaluated different techniques to overcome this problem, however, cell therapies, which consist in the use of resident cells of the tissue, such as chondrocytes or mesenchymal stem cells (MSCs) in order to regenerate the tissue, became the principal solution to repair articular cartilage. Moreover recently, another type of cell has been discovered to regenerate these defects, these cells are the Articular Cartilage Progenitor Cells (ACPCs). Another relevant factor that has been studied is how to stimulate these cells to use and integrate them as transplants for patients. Different growth factors have been assessed, however, in this project we focused on the Bone Morphogenic Protein 9 (BMP9) effects in the chondrogenic differentiation process of ACPCs. Other factors, such as cell growth environment and scaffold, which are also crucial to achieve articular cartilage regeneration, were also taken into account. Therefore, the main objective of this study was to explore the effects of the growth factor BMP9 on ACPCs and how it enhances their capacity to regenerate the articular cartilage.

1. INTRODUCTION

Articular cartilage is a highly specialized connective tissue in synovial joints, its main function is to lubricate and smoothen the joints in between bones for articulation and to facilitate the transmission of loads with a low frictional coefficient.[1] This hyaline cartilage lacks vascularization, lymphatics and nerves, and in comparison to other tissues, it has a low density of resident cells, the chondrocytes. It is mainly composed of water (68–65%) and an extensive extracellular matrix (ECM). The ECM contains a dense network of macromolecules with a dry weight basis of 60–85% Collagen, being 90–95% Collagen type II (Col-II) which provides high elasticity and strength, and 15–40% of proteoglycans, containing an 80% of Aggrecan, that contribute to maintain a high osmotic pressure while interacting with water.[2,3]

Articular cartilage features provide a wide low-friction and highly durable resistant surface to protect the joints, however, this tissue has a minimal regeneration and healing potential that might be related to the lack of blood vessels and the low cell density. Articular cartilage defects caused by traumatic injuries or pathological conditions can lead to progressive joint degeneration and, as articular cartilage has a low regenerative capacity, when these osteochondral defects are left untreated, they can lead to early Osteoarthritis (OA).[4]

OA is a chronic disease that is defined as an heterogenous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage and bone morphology.[5] While this progressive disease is usually associated to elder people, athletes or young individuals are susceptible to it as well. Although there are several risk factors apart from age that predispose an individual to suffer OA, articular cartilage damage by repetitive impact and loading usually leads to develop OA overtime in young adults and specially athletes.[6] Moreover, Steinwachs et al. report that in high-level athletes, the overall prevalence of knee articular cartilage lesions has been reported to be 36% to 38%.[7]

Nowadays, there are different techniques to treat OA. In severe cases, surgeries of the whole joint are performed. This procedure is just applied in serious cartilage lesions, nevertheless, the replacement of the tissue for a prosthetic device still fails to mimic the complex environment of the native cartilage tissue.[8] Currently, in those cases were the damaged cartilage area is localized, there are a few surgical approaches to overcome this problem.[7] The first one is microfracture, which consists in an arthroscopic marrow stimulation that forms a clot of fibrin and precursor cells from the bone marrow by drilling small holes through the articular cartilage tissue and into the subchondral bone. Under local biochemical and biomechanical factors, the bleeding generates from primitive scar tissue to fibrous-hyaline mixed cartilage tissue. The principal advantage of

this technique is the minimally invasive approach, nevertheless, the main disadvantage is the inferior mechanical properties of the new tissue, which limits the tissue durability and functionality.[9]

Another technique is Autologous Chondrocyte Implantation, based on tissue regeneration by isolated autologous chondrocytes. First, chondrocytes are harvested arthroscopically and then cultured. Second, the cell suspension is implanted in another surgical procedure under a periosteum flap or collagen membrane. Finally, after the cells have adhered to the defected area, the primary tissue evolves, and during approximately two years, matures until it becomes a repaired cartilage tissue under the local biochemical and biomechanical conditions. The main advantage of this procedure is that it can be used for larger defects than 2 cm^2, however, there are a few drawbacks on this technique, and the principal one is that more hyaline-like histologic quality seems to be essential for a long-term repairing tissue.[10]

Lastly, the Osteochondral Transplantation or Mosaicplasty, which uses cylinders harvested from low stress areas of the joint to adhere and repair defects from the same joint. The major difference of this technique in comparison to the previous ones is the maturation process of the used cells, which in this case is not necessary because the transplanted tissue is already hyaline cartilage, however, the principal disadvantages are that it can only address small defects and the lack of sufficient healthy cartilage available in patients.[10]

The exposed procedures show limited results with repairing articular cartilage, leading to fibrotic tissue which lacks the qualities of the native tissue, furthermore, even if they provide some relief to patients, these techniques fail in the durably repair of the cartilage.[11] As a result of an increased understanding of the structure and function of cartilage under both physiologic and pathologic conditions, new approaches for its regeneration are being studied. For these reasons, cell therapies have emerged as possible candidates. The main aim of these procedures is to repair damaged joint surfaces with functional replacement tissue. However, as this avascular tissue is unable to repair damage, choosing the best cell source and molecular cues to use is one of the principal challenges.

Chondrocytes, as previously exposed, have been used in cell-based therapies, nevertheless, this cellular type has some drawbacks when used as cell source. When these are expanded *in-vitro* and cultured in monolayers, they lose their chondrogenic phenotype and tend to dedifferentiate, which results in fibrous cartilage, rich in Coll-I and lacking the compressive strength and frictionless properties of the native articular cartilage.[8] Furthermore, the amount of chondrocytes in the native tissue is low, making their harvesting from the patient challenging as well.[11]

An alternative cell-type for cell-based therapy are mesenchymal stem cells (MSCs), which have been widely used during the last years in the regenerative medicine field. MSCs can be isolated from a variety of tissues (bone marrow, periosteum, synovium, adipose tissue, skin, adipose tissue, skeletal muscle, cord blood and amniotic fluid), have common phenotypic characteristics, an inherent proliferative capacity and immunomodulatory properties.[12] Furthermore, as this cell type express different stem-cell markers, after numerous expansions, it can be differentiated into different cell lineages, in our case, the chondrogenic lineage. However, MSCs tend to derive into osteochondral forms, resulting in an hypertrophic cartilage phenotype and calcification of the repaired zone, where the quality and durability of the repaired tissue is not ideal and it does not efficiently integrate with the surrounding tissue.[13,14]

In recent studies, Articular Cartilage Progenitor Cells (ACPCs) have emerged as another possible cell source for articular cartilage regeneration. These cells are characterized as resident, cartilage-specific, multipotent progenitor cells and they are found in the superficial zone of articular cartilage.[15] Some studies have identified this subpopulation of chondrocytes and have shown their stem cell-like qualities and chondrogenic potential. ACPCs maintain sex determining region Y-box 9 (Sox9) and Notch1 expression and telomere length following extensive culture expansion, so they can be expanded to relevant numbers without dedifferentiating and undergo trilineage differentiation into bone, cartilage or adipose tissue.[16] Moreover, ACPCs seem to be resistant to hypertrophy, due to the lack or low expression of RUNX2, the master transcription factor of chondrocyte terminal differentiation and formation of calcified tissue.[14] Hence, articular cartilage progenitor cells are proposed as a suitable candidate for improving cell-based tissue repair therapies for cartilage defects.[17]

In order to use these cells for cartilage regeneration, research has focused on the use of different growth factors to stimulate their chondrogenic potential.[17] Factors that promote chondrogenesis both *in vivo* and *in vitro* include Bone Morphogenic Proteins (BMPs), Transforming Growth Factor Beta (TGFβ1), and insulin-like factors. Chondrocytes and MSCs chondrogenic differentiation conditions have been widely described, containing TGFβ1, dexamethasone, ascorbate and insulin.[18] Nevertheless, further investigation about the optimal chondrogenic medium for ACPCs needs to be done. As previously mentioned, TGFβ1 is one of the principal growth factors used in MSCs chondrogenic differentiation medium. TGFβ1 signals through the activin receptor like kinase 5 (Alk-5), a type I receptor of the TGFβ superfamily (TGFBRI) that forms a complex with the type II receptor (TGFBRII).[19] This binding triggers the phosphorylation of the receptor and the subsequent activation of the transcription factors Smad 2/3 in the canonical TGFβ pathway.[20] However, TGFβ1 effects mainly showed a lack of potency in the ACPCs

chondrogenic differentiation process, thus indicating that there might be other potent chondrogenic factors for this type of cells.[21,22] For this reason, in this project we focus in the development agent BMP9, which recently has emerged as an alternative to enhance ACPCs chondrogenic potential, promoting cartilage matrix production and mature-like cartilage morphogenesis.[21,23] This growth factor signals through the activin receptor-like kinase 1 (Alk-1), a type I receptor of the TGFβ superfamily (ACTRL1) that transduces the growth factor signal into the cell through the transcription factors Smad 1/5/8, nevertheless, Alk-1 can also be activated, with less affinity, by TGFβ1, thus indicating a crosstalk between these two growth factors and the receptor. Moreover, BMP9 has been found to induce hypertrophy-like state on chondrocytes[24], suggesting that, as previously mentioned, this growth factor might have a different effect in each type of cell. TGFβ1 and BMP9 have been mainly studied in endothelial cells and chondrocytes, for this reason, in this project we tried to define the optimal pathways for chondrogenic differentiation and organized extracellular matrix production, under the possible BMP9 chondrogenic effects, on ACPCs.

Another factor commonly used to induce human MSCs into chondrogenic differentiation is dexamethasone. However, this synthetic glucocorticoid could have a negative impact in this process, as it modulates different growth factor functions disturbing the chondrogenic process and deriving cells into adipogenic differentiation.[25] Some studies state that this glucocorticoid might distinctly effect on chondrogenesis depending on the cell type, the combined growth factors and the culture system used.[26] In previous experiments of this project, the dexamethasone effect on ACPCs was also assessed, and consequently, its impact to the BMP9 signaling. Nevertheless here, and since previous results showed negative effects of this glucocorticoid towards BMP9, dexamethasone was only used in combination with TGFβ1.

However, not only cell therapies and growth factors have been used to enhance the articular cartilage regeneration, a wide variety of tissue engineered 3D scaffolds have been studied to promote this effect. In the regenerative medicine and tissue engineering field, the combination of the different factors such as cell source, growth factors and 3D system used are key to offer promising potential solutions to heal and restore the functionality of articular cartilage. For this reason, an appropriate culture environment is necessary to unlock the regenerative potential of ACPCs. Biomaterials used in articular cartilage cell therapy are important to enhance the chondroprogenitor capacity, because they act as cell delivery vehicles and provide a supportive environment for cartilage matrix to develop.

It is well established that during cartilage development, the microenvironment gradually transitions from one rich of cell-to-cell interactions to one dominated by cell-ECM (extracellular matrix) interactions[27], and as just mentioned, microenvironment mechanical properties directly affect stem cell fate and behavior.[28,29] The principal molecules responsible for cell-to-cell interactions are N-cadherins, transmembrane proteins in charge of modulating the cell adhesion that play a key role during the mesenchymal condensation and chondrogenesis.[30] Furthermore, recent studies have shown that hydrogels mimicking these developmentally relevant matrix features and N-cadherin adhesions, enhance the MSCs chondrogenesis. Specifically, the addition of an optimized synthetic peptide which mimics the evolutionary conserved 'HAVDI' adhesive sequence from the extracellular N-cadherin domain (ECD1), generates N-cadherin-like features inside the hydrogels. Previous analysis were done encapsulating MSCs inside of modified Hyaluronic Acid hydrogels (HA) containing N-cadherin mimetic peptides, therefore, further research about this type of mimetic hydrogels combined with ACPCs is needed.

The principal aim of this study was to test the hypothesis that the growth factor BMP9 enhances the chondrogenic potential in ACPCs. In order to explore this effect, in this study we blocked the cell receptor ALK-1, to first, check if BMP9 acts through this receptor in ACPCs, and second, to prove the main effect of BMP9 in the chondrogenic process comparing ACPCs and MSCs. Moreover, BMP9 was also used in the chondrogenic differentiation of three different ACPCs donors to test its effect. This boosting effect was also assessed in two different types of 3D scaffolds for chondrogenic differentiation: Norbornene-modified Hyaluronic Acid hydrogels bearing n-cadherin mimetic peptides (+HAVDI) and Polycaprolactone 3D printed meshes. ACPCs were encapsulated and differentiated in NorHA (+HAVDI) hydrogels, where the hypothesis that hydrogels containing the mimetic peptide that stimulates cell-to-cell contacts will increase the chondrogenic differentiation of ACPCs, as normally experienced by this cells during the mesenchymal condensation phase in the limb development, was addressed. ACPCs were also differentiated inside of 5 mm cell-laden PCL meshes to test this structure as a suitable cell-laden articular cartilage implant. In both experiments BMP9 was added to the differentiation medium. Finally, the hypothesis that the three possible cell candidates for articular cartilage regeneration, chondrocytes, MSCs and ACPCs, could have diverse amounts of ALK-1 and ALK-5 receptors, thus explaining the different response to the two growth factors, BMP9 and TGFβ1, was also evaluated.

2. MATERIALS AND METHODS

2.1. Cell culture: MSC, ACPC, Chondrocytes

Bone marrow derived human mesenchymal stem cells (MSCs) were taken from vials containing 2 million cells (donor #20, passage 3). 250.000 cells were seeded per T175 culture flask and expanded in MSCs expansion medium containing Dulbecco's modified Eagle medium high glucose + pyruvate (DMEM, 31966, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 1% penicillin and streptomycin (Life Technologies) and 1 ul/mL basic fibroblast growth factor (b-FGF, Peprotech). Human Articular Cartilage Progenitor Cells (ACPCs) were taken from vials containing 1,5 million cells (donor OA2, AM4, AM2, passage 3 and 4). 250.000 cells were seeded per T175 culture flask and expanded in chondroprogenitor expansion medium containing DMEM supplemented with 10% FBS, 0.2 mM ascorbic acid-2-phosphat, 1% penicillin and streptomycin, 5 ul/mL b-FGF and 1% MEM non-essential amino acids solution (NEAA, Gibco). Both type of cells had their media refreshed every three days. Human Chondrocytes were taken from vials containing 1 million cells (donor AM2, passage 0-1). 250.000 cells were seeded per T175 culture flask and expanded in DMEM, supplemented with 10% FBS and 1% penicillin and streptomycin. Chondrocytes medium was first refreshed one week after the seeding, the following medium changings were three days. All type of cells were cultured at 37ºC and 5% CO_2 in the incubator.

2.2. Chondrogenic differentiation

To differentiate ACPCs and MSCs into Chondrocytes they were cultured in a basal chondrogenic medium containing DMEM high glucose + pyruvate, 1% insulintransferrin-selenous acid (ITS+ Premix, Corning), 0.2 mM ascorbic acid-2-phosphat, 1% penicillin and streptomycin and 1% 1M HEPES (Gibco). 100nM dexamethasone (4ul/100mL of media) was also added to the basal media in certain conditions where the growth factor TGF-β1 was also present. The main growth factors used in the differentiation process were BMP9 (100ng/mL) (PeproTech) and TGF-β1 (10ng/mL) (PeproTech), both also used in synergy (50ng/mL BMP9 and 1ng/mL TGF-β1).

2.3. Pellet formation

For three-dimensional pellet culture, after cell trypsinization, ACPCs and MSCs were resuspended in an arbitrary amount of expansion medium and counted in an automatic cell counter. Once cells were counted, these were resuspended in the chondrogenic differentiation basal medium, and then added specific growth factors for different conditions. Pellets were seeded in a cell density of 250.000 cells per well in a low-attachment 96-well plate (Costar, #7007), then

centrifugated at 1500 rpm for 5 minutes without brake and acceleration. Differentiation medium was refreshed every three days and plates were incubated for 28 days at 37ºC and 5% CO_2. Samples were harvested on day 1 (for qPCR) , day 7 and day 28.

2.4. Blocking ALK-1 receptor

To prove if activin receptor-like kinase 1 (ALK-1) is the cell receptor of the growth factor BMP9 in human MSCs and ACPCs, this was blocked by the blocker K02288 (Shelleckchem) and used at 1 μM. The experiment consisted in three groups, medium with blocker added just on the first day, medium without blocker and medium containing always blocker, and four different conditions, BMP9 (100ng/mL), TGF-β1 (10ng/mL) synergy of BMP9 and TGF-β1 (50ng/mL BMP9 and 1ng/mL TGF-β1) with and without dexamethasone (1mg/mL).

2.5. SDS-PAGE and Western Blot

To perform the western blot, after culturing MSCs, ACPCs and Chondrocytes in their specific expansion medium mentioned before, cells were trypsinized, collected and counted. Aliquots of 0,5, 1 and 2 million cells in PBS of each type of cells were made. Cells were lysed in 10x RIPA buffer (Abcam, ab156034,) for 20 minutes at 4ºC. Protein concentration was determined by BCA protein assay (Pierce, #23225,) then denatured samples with Laemmli buffer 4X (loading buffer) 5 minutes at 95ºC. 25 μg of protein for each sample were load on a 5-12% TRIS-glycine SDS-Polyacrylamide gel and run for 1h at 150V and 50mA. 5 μg of protein ladder (Thermo Scientific, #26619) were also loaded into the gel. Proteins were then transferred into a PVDF membrane (Immobilon), previously activated in 96% ethanol, at 100V and 400mA for 1,5h, blocked with TBST-MILK 5% buffer for 1h, and left o/n in primary antibodies for ALK-1 (1:1000, R&D Systems, #AF370) or ALK-5 (1:1000, R&D Systems, #AF3025) at 4º in the roller bench. Next morning, membranes were exposed to Polyclonal Rabbit Anti-Goat secondary antibodies conjugated with horseradish peroxidase (HRP) (1:2000, Dako, #P0160) for 1h and left in the roller bench. Finally, protein bands were detected using Dura substrate (SuperSignal, #34075) on a ChemiDoc MP Imaging System (Bio-Rad).

2.6. ACPCs encapsulation in Norbornene-modified Hyaluronic Acid hydrogel gel

Chondroprogenitor cells were encapsulated in Norbornene-modified Hyaluronic Acid hydrogels (NorHa), containing a N-cadherin mimetic peptide sequence (HAVDIGGGC, ~869.95 g/mol, peptides with cysteine residues at the C-terminal, GenScript). Gels were crosslinked via cell-friendly thiol-ene click chemistry by exposure to visible blue light (405 nm), in presence of the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) and the crosslinker

dithiothreitol (DTT). NorHa hydrogels with a scrambled and non-functional peptide sequence, were used as control. As described before, ACPCs were first, trypsinized, collected and counted. Then cells were resuspended in a specific volume of Phosphate Buffered Saline (PBS) and finally mixed and embedded in the macromer solution at a concentration of 20 million cells/mL. The mix was casted in 5 x 2 mm silicone molds and crosslinked by the irradiation of visible blue light for 8 minutes at an intensity of ~10 mW/cm^2. Once scooped out from the silicon molds, ACPC and MSC-laden hydrogels were cultured in the basal chondrogenic differentiation medium mentioned before with specific growth factors for each conditions (100ng/mLBMP9, 10ng/mL TGFb1, 1 shot of 100ng/mL BMP9) in a 48-well plate. Medium was refreshed every three days and plates were incubated for 28 days at 37ºC and 5% CO_2. Samples were harvested on day 1 (for qPCR) , day 7 and day 28. Cell-free gels were used as control.

2.7. ACPCs in polycaprolactone meshes

Chondroprogenitor cells were seeded on top of a 5mm circled polycaprolactone meshes (PCL, Purasorb PURASORB PC 12, Corbion PURAC) fabricated by Melt Electrowriting (MEW) with a closed surface achieved by depositing MEW fibers with an inter fiber distance of 100 μm in a box-pattern in an alternating 0 μm - 45 μm - 90 μm - 135 μm direction. To increase hydrophilicity, meshes were hydrolyzed by submerging them 30 minutes in 1M sodium hydroxide solution, followed by 3 washes of MiliQ water of minimum 10 minutes. In a sterile environment, meshes were then submerged in 70% ethanol for 20 minutes, and followed by 3 washes for 5 minutes of MiliQ. Finally, meshes were left air-dried under UV-light. Meshes were put inside rounded silicon molds that were placed inside a 48-well plate previously. The silicon molds were also sterilized earlier, by ethanol 70%, 3 washes of 5 minutes of MiliQ and UV-light. As described before, ACPCs were first, trypsinized, collected and counted. Then cells were resuspended in chondrogenic differentiation media containing specific growth factors for each condition (100ng/mL BMP9 and 1 shot of 10ng/ml BMP9 only for day 1) in order to have a cell density of 5 million cells/120 ul, which is the maximum volume that one mesh can contain. ACPCs were then seeded inside the silicon mold on top of a mesh or only cells without a mesh as scaffold. Medium was refreshed every three days and plates were incubated for 28 days at 37ºC and 5% CO_2. Samples were harvested on day 7 and day 28.

2.8. Biochemical Analysis: PicoGreen and DMMB assays

MSCs and ACPCs pellets were harvested to measure DNA and Glycosaminoglycans (GAGs) content at day 7 and day 28 of culture (n=3). Samples were digested in 250 μg/mL of papain o/n at 60ºC. Once the digestion was done, DNA content was assessed by a Quant-iT PicoGreen

dsDNA kit (Life Technologies, The Netherlands). A stock solution of λDNA at a concentration of 1μg/mL (5 μL λDNA in 495 μL TE buffer) was made and a standard curve was prepared. Samples were diluted differently depending on the culture conditions and the harvest day in 1X TE buffer. Then, 100 μL of each diluted sample was transferred to a 96-well plate and the PicoGreen reagent was diluted 200x in TE buffer and added 100 μL to each well. After incubating the well plate for 5 minutes in the dark, fluorescence at 485 nm excitation and 520 nm emission was measured. GAGs content was also quantified by the dimethyl methylene blue assay (DMMB, Sigma-Aldrich, The Netherlands). The standard curve of Chondroitin Sulphate was made and the samples were diluted differently depending on the culture conditions and the harvest day in PBS-EDTA. Then, 100 μL of each diluted sample was transferred to a 96-well plate, finally, 200μL DMMB staining solution was added to each well and the plate was measured at 525nm and 595nm. Cell-laden NorHa hydrogels and cell-laden PCL meshes DNA and GAGs amount were quantified following the same protocols, however, NorHa hydrogels were also digested in 1mg/mL of hyaluronidase (Sigma, #H2126) in the initial step.

2.9. Alkaline Phosphatase assay (ALP):

After day 7 and day 28 of culture, samples were harvested to perform an Alkaline Phosphatase assay (n=3). Samples were first stored at -80ºC with 250 μL MPER buffer. After a few cycles of freeze, thaw, samples were crushed with special pistons stored in 70% ethanol. Then the pNPP and Tris-Buffer tables (Sigma-Aldrich) tablets were dissolved in 20 mL PBSO and the ALP enzyme standard curve was prepared. 25 ul of the samples and the standard curved were added to the 96-well plate, finally, 50 ul of the pNPP solution was added to each well and the plate was read at 405nm and 655 nm.

2.10. Histological analysis: Safranin-O, Collagen-I, Collagen-ll

After day 7 and day 28 of culture, samples were harvested to perform the histological analysis of the main components of the cartilage ECM (n=3). Cell pellets, cell-laden hydrogels and cell-laden meshed were fixed for a minimum of 24h in Phosphate buffered formaldehyde 4% (Klinipath) before dehydration. Cell pellets were first embedded in Alginate (Fisher Scientific), followed by the same dehydration process as cell-laden hydrogels and cell-laden meshes. Samples were then embedded in paraffin in histology cassettes, and once these were cooled down, 5 µm slices were made with the microtome (Leica) and then processed for different staining.

Safranin O and Fast Green staining was used to visualize GAGs content. Slides were first deparaffinated, then soaked for 5 minutes in Weigert's Haematoxylin solutions (Klinipath), rinsed

in running tap water for 10 minutes and rinsed by distilled water. Then slides were incubated in 0,4% Fast Green FCF solution (MP Biomedicals) for 4 minutes followed by a series of washing with 1% Acetic Acid (Sigma-Aldrich) until no stain runs through. Finally they were incubated in 0,125% Safranin-O (Sigma-Aldrich) for 5 minutes, followed for fast changes of 96% ethanol, absolute ethanol and two last changes of xylene to fix.

Both collagen type I and II were visualized using immunohistochemistry. Slides were deparaffinized in xylene, hydrated in decreasing concentrations of ethanol solutions and endogenous peroxidases were blocked with peroxidase solution (0.3%), Pronase (1 mg/mL, Roche) and hyaluronidase (10 mg/mL, H2126, Sigma Aldrich) were applied for antigen retrieval for 30 minutes at 37 °C. Sections were blocked with bovine serum albumin (5% BSA-PBS) for 60 minutes at room temperature. Primary antibodies for collagen type I (Abcam, #ab138492,) and type II (DSHB, #II-II6B3) were used for incubation o/n at 4 °C. Appropriate IgGs (Dako) were used as negative controls corresponding to the primary antibodies used. Then, sections were incubated with a secondary antibody (Dako, #P0447) conjugated with HRP for 1 hour at room temperature. Sections were incubated with 3,3-diaminobenzidine-horseradish peroxidase (Sigma Aldrich) in order to develop the staining. Cell nuclei were counterstained with Mayer's hematoxylin solution, followed by fast changes of 96% ethanol, absolute ethanol and two last changes of xylene to fix. All the slides were examined using a bright-field microscope (BX51, Olympus) and photomicrographs were taken.

2.11. Gene expression of cartilage markers: quantitative-PCR

Gene expression of ACPCs and MSCs pellets, cultured under different chondrogenic differentiation mediums, was analyzed by qPCR samples after 1 and 7 days of culture (n=3). Pellets were harvested and mechanically lysed with RLT Lysis Buffer (Qiagen #79216), and total RNA was isolated using RNeasy Mini Kit (Qiagen, #74104). A Superscript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, #11736-059) was used for the cDNA synthesis. The relative expression levels of collagen type X, Notch1, RunX2, Cadherin and Sox9 were analyzed and normalized to the housekeeping gene HPRT1, and this was calculated using a PCR miner algorithm.

2.12. Immunofluorescence: Col II, Col VI, DAPI, Phalloidin-TRICT

Immunofluorescence was performed on samples harvested after 7 and 28 days of culture, that were first fixed o/n in phosphate buffered formaldehyde 4% (Klinipath) at 4ºC. Samples were washed 3 times for 5 minutes in PBS-glycine 0.1M, then washed 10 minutes in PBS-Tween, then

left in PBS-BSA at RT for 1 hour and then washed 3 times for 5 minutes in PBS-Tween. Samples were then incubated o/n at 4ºC with primary antibodies, Col-II (1:1000, DSBH, #II-II6B3) and Coll-VI (1:5, DSBH, #5C6-s) diluted in BSA-PBS 5%. The following day, samples were washed 3 times for 5 minutes in PBS-Tween and the secondary antibody was added (Alexa 488 anti-mouse, Invitrogen, #A10680), diluted in BSA-PBS 5% at RT for 1 hour. Samples were then washed 3 times for 5 minutes in PBS-Tween, and DAPI (100X, Sigma Aldrich, D9542) and Phalloidin (1:200x, MERCK) were added for 30 minutes in the dark. Finally, samples were washed 3 times in PBS and left at 4ºC. Confocal (Leica) pictures were taken at 20x magnification.

2.13. Mechanical testing: DMA

The mechanical properties of the samples of the cell-laden polycaprolactone meshes were studied after 7 and 28 days of culture (n=3) in an unconfined uniaxial compression test. Samples were washed with PBS and compressed at -20%/minute strain rate with a Dynamic Mechanical Analyzer (DMA Q800, TA Instruments). The compressive modulus (Young's modulus) was obtained from the slope of the linear section of the stress/strain curves.

2.14. Statistical Analysis

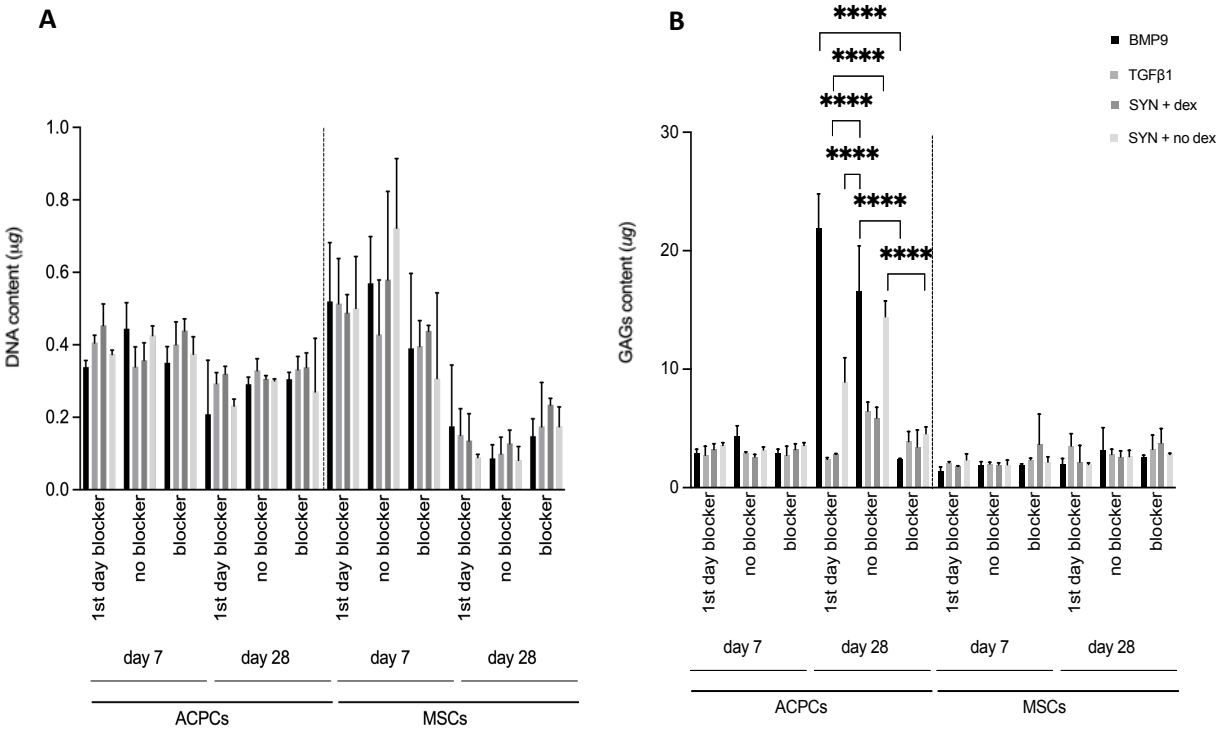
All experiments were performed in a minimum of three replicates (n=3). Statistical analysis and graph representation was performed using GraphPad Prism 8 software. Results are expressed as mean (M) and standard deviation (SD). For multiple comparisons of the results, these were analyzed via a two-way ANOVA, with a Bonferroni post hoc test, $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Blocking ALK1-receptors in ACPCs and MSCs pellets

Biochemical analysis: Picogreen, DMMB

DNA quantification (Fig. 1 A) showed that, in ACPCs samples, the number of cells slightly decreased not significantly for all conditions after 28 days of culture, independently of the ALK-1 blocker addition. However, we observed that MSCs, at day 7, had a higher amount of cells, and these decreased significantly after 28 days of culture in all the groups and conditions. In terms of neocartilage deposition (Fig. 1 B), there was an increment of GAGs content after 28 days of culture in ACPCs samples that had the ALK-1 receptor blocked just the first day of culture or were not blocked. Specifically, the higher GAG production was observed in the BMP9 and Synergy without dexamethasone conditions. The GAGs production after 28 days of MSCs remained constant in the three groups and conditions. The increment in GAGs/DNA (Fig. 1 C) in the ACPCs samples, on the one hand was significantly higher in both, blocker on the first day and no blocker groups, with the BMP9 and Synergy without dexamethasone conditions after 28 days of culture. On the other hand, the blocked group showed the highest increment of GAGs/DNA in the synergy with dexamethasone condition. These increments were significantly higher in the group blocked on the first day and the non-blocked group compared to the blocked group for the BMP9 condition. Finally, MSCs samples had a non-significant increment of GAGs/DNA after 28 days of culture for the three groups and the different conditions.



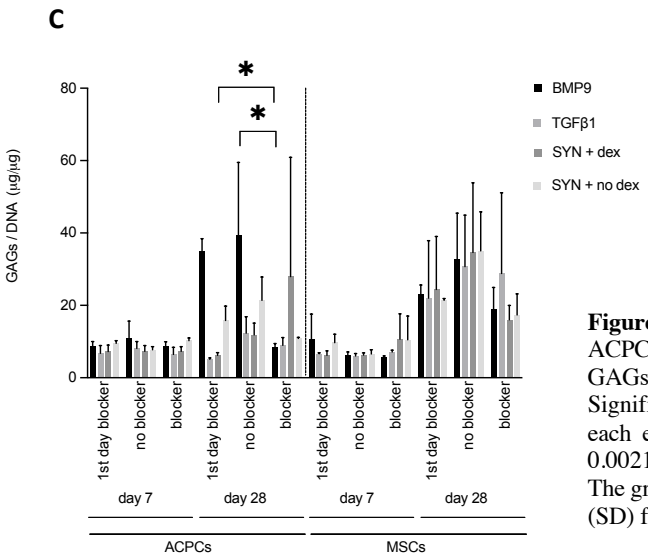


Figure 1. Quantification of DNA (A) and GAGs (B) in ACPCs and MSCs pellets after 7 and 28 days of culture. GAGs quantification was normalized to DNA content (C). Significant difference between columns is shown below each end of lines (* means $p < 0.0332$, ** means $p < 0.0021$, *** means $p < 0.0002$, **** means $p < 0.0001$). The graph shows the mean (M) with the standard deviation (SD) for each group (n=3).

Alkaline Phosphatase levels

The amount of alkaline phosphatase levels was also assessed in ACPCs and MSCs samples after 7 and 28 days of culture to evaluate the hypertrophy levels of our samples. We observed a significant increment of ALP signal in ACPCs. In the group of ALK-1 blocker added just at day one, we found the higher values in the TGFβ1 and Synergy with dexamethasone conditions. We also observed an increment of this two conditions in the no blocker group. However, in the blocked group, the increment of ALP signal was homogeneous for all the different conditions. The levels of ALP signal in the different MSCs groups got maintained during the 28 days of culture.

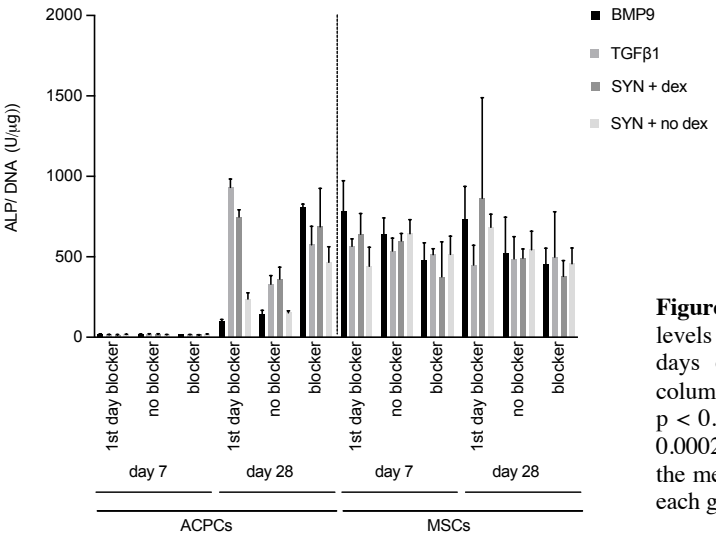


Figure 2. Quantification Alkaline Phosphatase levels in ACPCs and MSCs pellets after 7 and 28 days of culture. Significant difference between columns is shown below each end of lines (* means $p < 0.0332$, ** means $p < 0.0021$, *** means $p < 0.0002$, **** means $p < 0.0001$). The graph shows the mean (M) with the standard deviation (SD) for each group (n=3).

3.2. Western blot: Detection of ALK1 and ALK5 in Chondrocytes, MSCs and ACPCs

In order to detect the presence of the growth factors cell membrane receptors ALK-1 and ALK-5, both having a molecular weight of 56 kDa, we conducted a Western Blot analysis of MSCs, ACPCs and Chondrocyte samples. On the one hand, ALK-1 expression (Fig. 3 A) was observed in the three type of cells at 56 kDa, however, the expression in chondrocytes appeared to be stronger than ACPCs and MSCs. On the other hand, ALK 5 expression (Fig. 3 B) was stronger in MSCs compared to the other two type of cells.

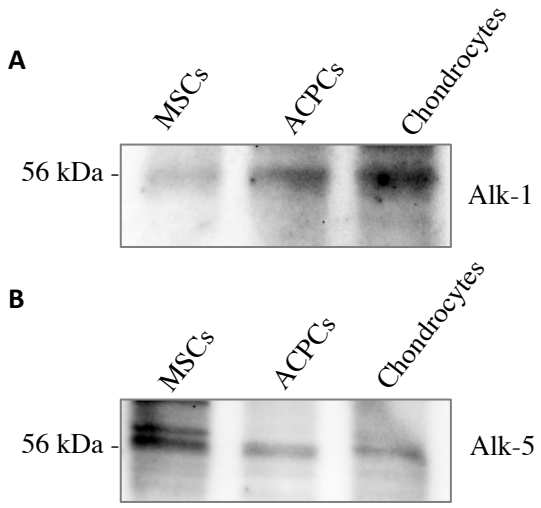


Figure 3. Western Blot analysis of ALK-1 and ALK-5 cell membrane receptors in MSCs, ACPCs and Chondrocytes. For the Western Blots, 25 ug of protein from the each type of cells was loaded. Samples were lysed and immunoblotted as described in materials and methods. Positions of molecular weights are indicated on the left side of the figures.

3.3. Comparing BMP9 effect in three different ACPCs donors

Biochemical analysis: Picogreen, DMMB

DNA quantification (Fig. 4 A) showed that the number of cells for both, the different donors of ACPCs and MSCS, decreased after 28 days of culture, with the exception of the TGFβ1 condition, which maintained a constant number of cells for the three different ACPCs donors. Regarding the neocartilage ECM deposition (Fig. 4 B), we observed a significant increment in the 3 ACPCs donors with BMP9 and one shot of BMP9 conditions, however, there is not a significant difference in between this two groups. The amount of GAGs deposition in the MSCs samples remained constant after 28 days of culture. Finally, the increment in GAGs/DNA (Fig. 4 C) was significantly higher in the 3 different ACPCs donors when cultured under BMP9 and one shot of BMP9 conditions.

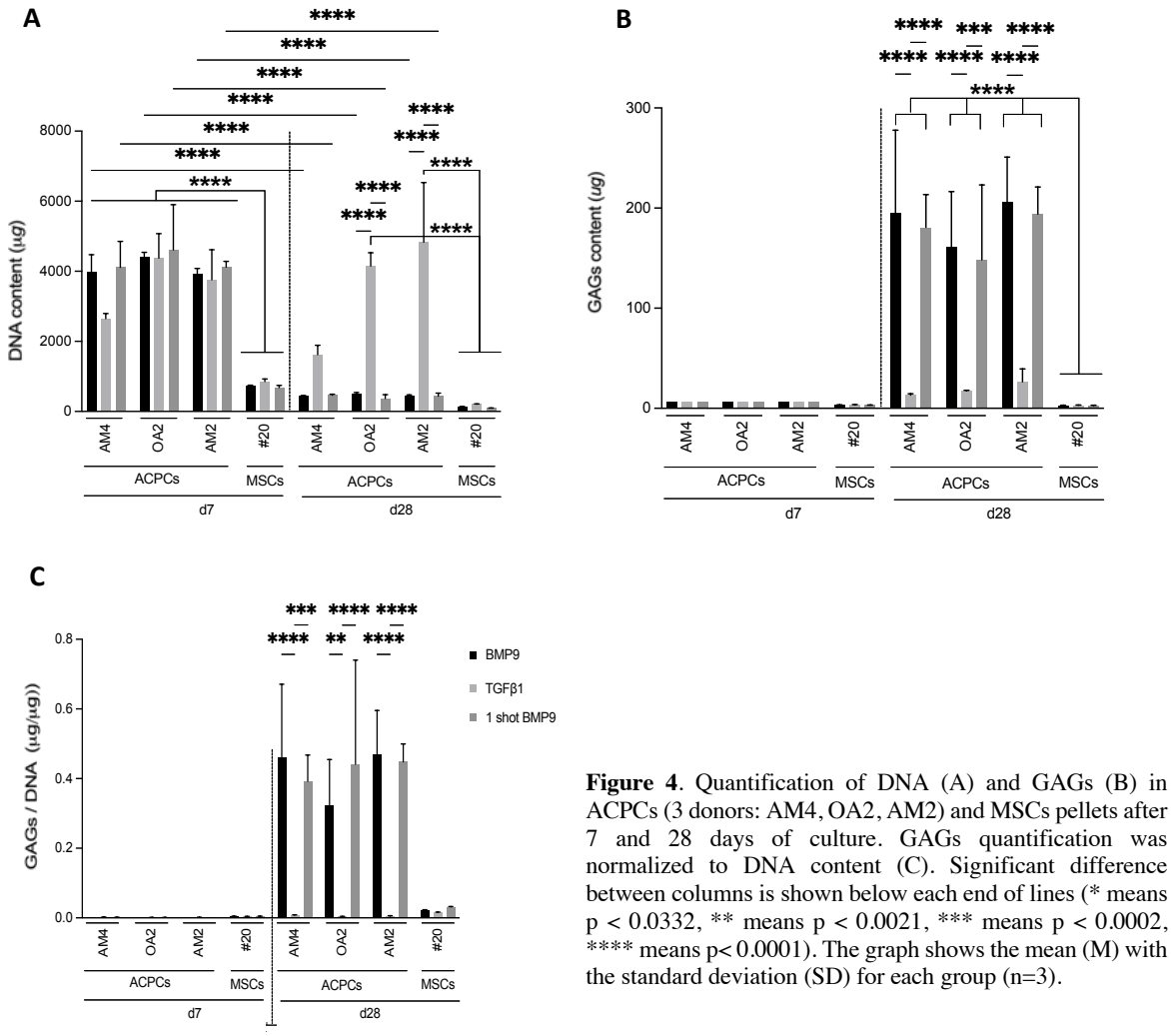


Figure 4. Quantification of DNA (A) and GAGs (B) in ACPCs (3 donors: AM4, OA2, AM2) and MSCs pellets after 7 and 28 days of culture. GAGs quantification was normalized to DNA content (C). Significant difference between columns is shown below each end of lines (* means $p < 0.0332$, ** means $p < 0.0021$, *** means $p < 0.0002$, **** means $p < 0.0001$). The graph shows the mean (M) with the standard deviation (SD) for each group (n=3).

Alkaline Phosphatase levels

The level of hypertrophy of our samples was also assessed by an Alkaline Phosphatase test. We observed a significant increment of these levels in all the ACPCs donors when cultured in BMP9 and one shot of BMP9. Results also showed a significant increment of ALP levels in MSCs.

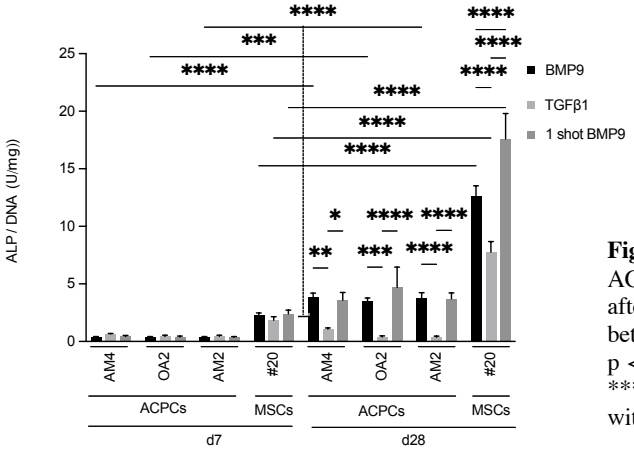


Figure 5. Quantification Alkaline Phosphatase levels in ACPCs (3 donors: AM4, OA2, AM2) and MSCs pellets after 7 and 28 days of culture. Significant difference between columns is shown below each end of lines (* means $p < 0.0332$, ** means $p < 0.0021$, *** means $p < 0.0002$, **** means $p < 0.0001$). The graph shows the mean (M) with the standard deviation (SD) for each group (n=3).

Histological assessment: Safranin-O

Aiming to analyze neo-cartilage ECM synthesis, qualitative observation of GAGs was performed on histological sections. In samples containing BMP9 in the differentiation medium, at day 7, OA2 (Fig. 6 B) and AM2 (Fig. 6 C) ACPCs donors showed a slightly higher GAGs production than AM4 donor (Fig. 6 A). At day 28 (Fig. 6 D-F), these ACPCs pellets went into chondrogenic differentiation and GAGs production was appreciated in the staining of the 3 different donors.

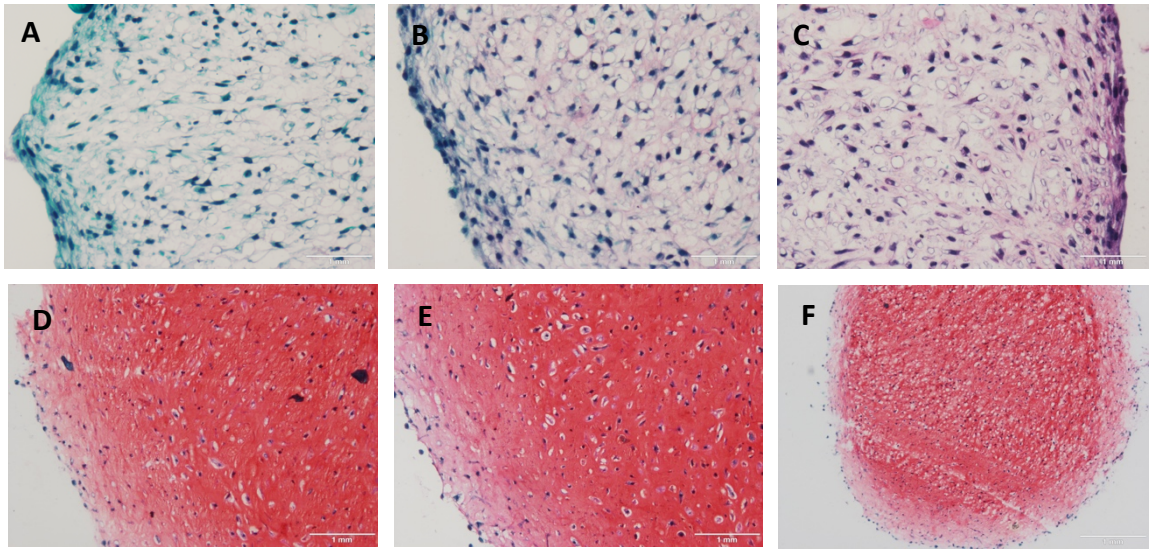


Figure 6. Safranine O staining at day 7 (A-C at 20x) and day 28 (D-F at 10x) of ACPCs pellets: AM4 donor (A, D), OA2 donor (B, E), AM2 donor (C, F) all cultured with BMP9 in the differentiation medium.

Pellets cultured with TGFB1 in the chondrogenic differentiation medium, at day 7 (Fig 7 A-D) the MSCs sample (Fig. 7 D) showed a higher positivity for GAGs signal compared to the other three ACPCs donors. When comparing these three ACPCs donors, OA2 (B) and AM2 (C) showed a slight higher ECM synthesis. After 28 days of culture, the three ACPCs donors showed diverse amounts of GAGs synthesis (Fig. 7 E-G), being AM2 donor the sample that showed a higher positivity for GAGs.

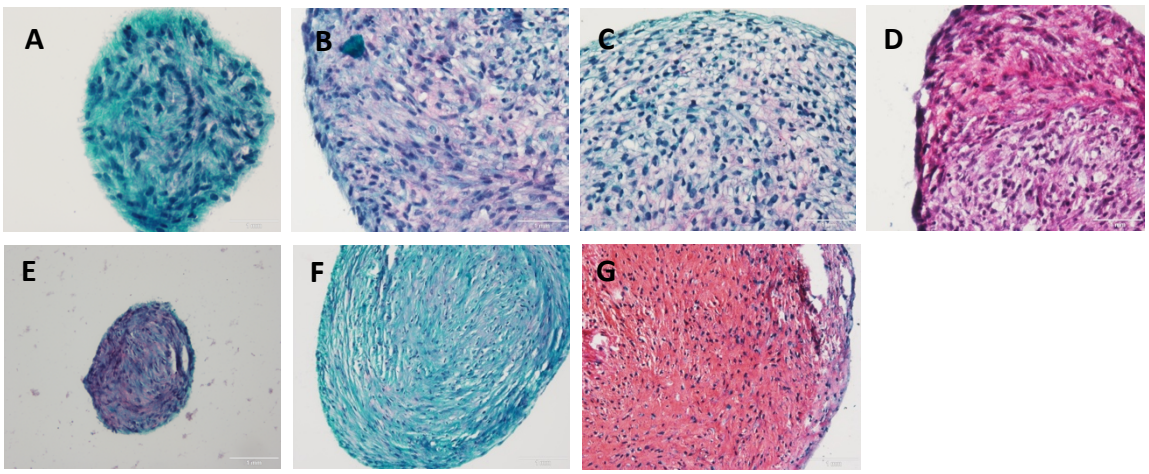


Figure 7. Safranine O staining at day 7 (A-D at 20x) and day 28 (E-H at 10x)) of ACPCs pellets: AM4 donor (A, E), OA2 donor (B, F), AM2 donor (C, G), and MSCs pellets (D) all cultured with TGFβ1 in the differentiation medium.

The last condition assessed was a single shot of BMP9 on the first day of the chondrogenic differentiation of the pellets. At day 7, we observed the same level of positive staining in all three ACPCs donors (Fig. 8 A-C). When these were compared to the MSCs sample, this one appeared to be lower in ECM synthesis (Fig. 8 D). ACPCs pellets went through chondrogenic differentiation and showed a high production of GAGs at day 28 of culture (Fig 11. E-F), there were no visible differences between donors. However, MSCs pellets did not show a positive signal for GAGs (Fig. 8 H).

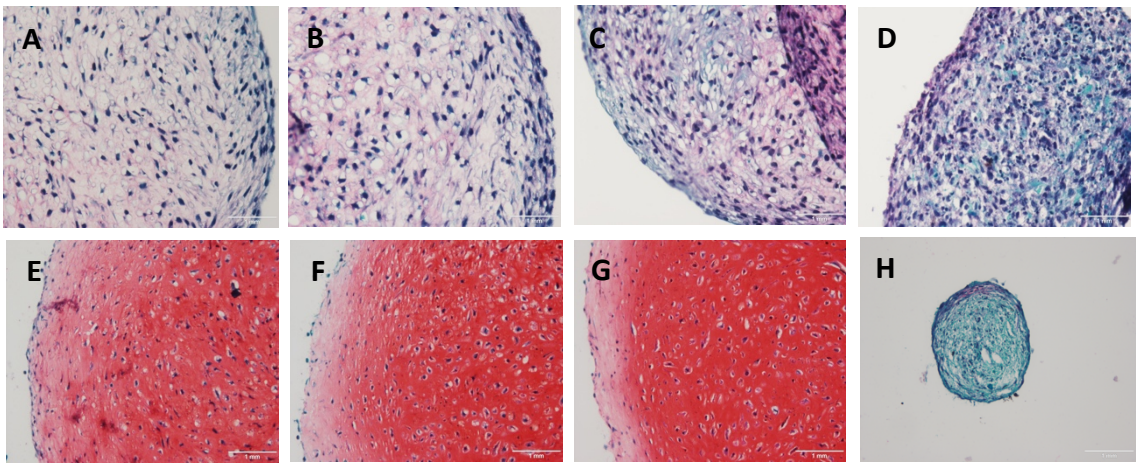


Figure 8. Safranine O staining at day 7 (A-D at 20x) and day 28 (E-H at 10x)) of ACPCs pellets: AM4 donor (A, E), OA2 donor (B, F), AM2 donor (C, G), and MSCs pellets (D, H) all cultured with one shot of BMP9 in the differentiation medium.

Histological assessment: Collagen-II and Collagen-I

Since collagen type II is one of the major components of the articular cartilage ECM, immunohistological analyses for this protein were performed. In the pellets cultured with BMP9 in the differentiation medium, we observed a slight positive signal Col-II in all 3 different ACPCs donors at day 7 (Fig. 9 A-C). The presence was considerably higher after 28 days of culture in the 3 donors (Fig. 9 D-F).

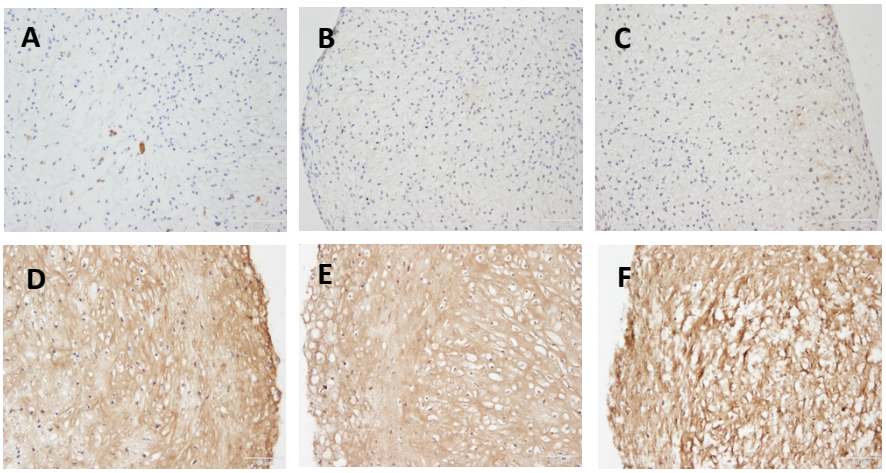


Figure 9. Collagen II staining at day 7 (A-C at 10x) and day 28 (D-F at 10x) of ACPCs pellets: AM4 donor (A, D), OA2 donor (B, E), AM2 donor (C, F),) all cultured with BMP9 in the differentiation medium.

In the samples cultured with TGFβ1, Col-II was barely detected at day 7 (Fig.10 A-D), although a slight positive signal was observed in the external part of the pellets. The highest amount of Col-II was detected in the ACPCs donor AM2 (Fig. 10 G). The other two donors showed lower Col-II signals.

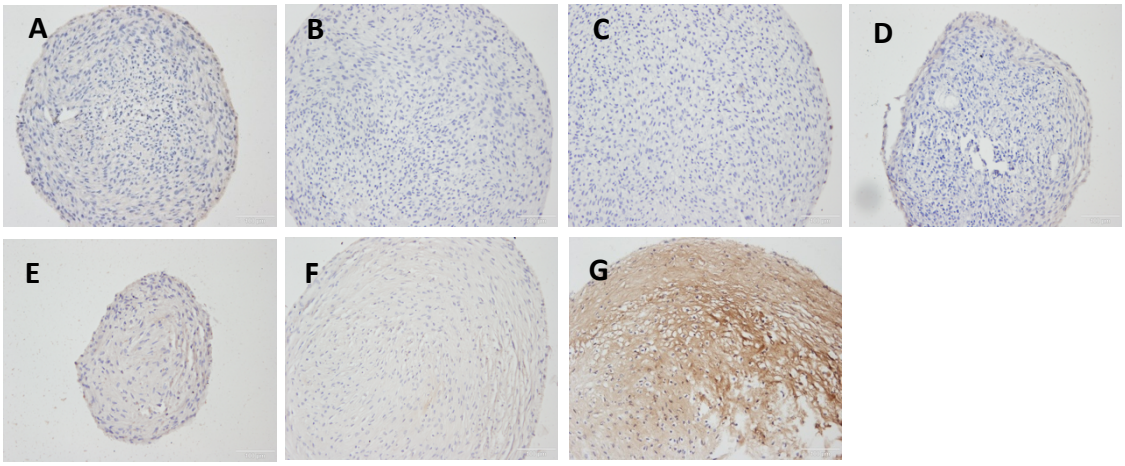


Figure 10. Collagen II staining at day 7 (A-D at 10x) and day 28 (D-F at 10x) of ACPCs pellets: AM4 donor (A, D), OA2 donor (B, F), AM2 donor (C, F),) and MSCs pellets (D), all cultured with TGFβ1 in the differentiation medium.

In samples harvested from medium containing just one shot of BMP9 in the first day of culture, Col-II presence was mainly detected in the three ACPCs donors, at day 7 (Fig. 11 A-B) with a slight signal spread all over the sample, and a stronger and homogeneous positive signal after 28 days of culture (Fig. 11 D-F). MSCs samples vaguely expressed any positive signal, neither at day 7 or day 28 of culture.

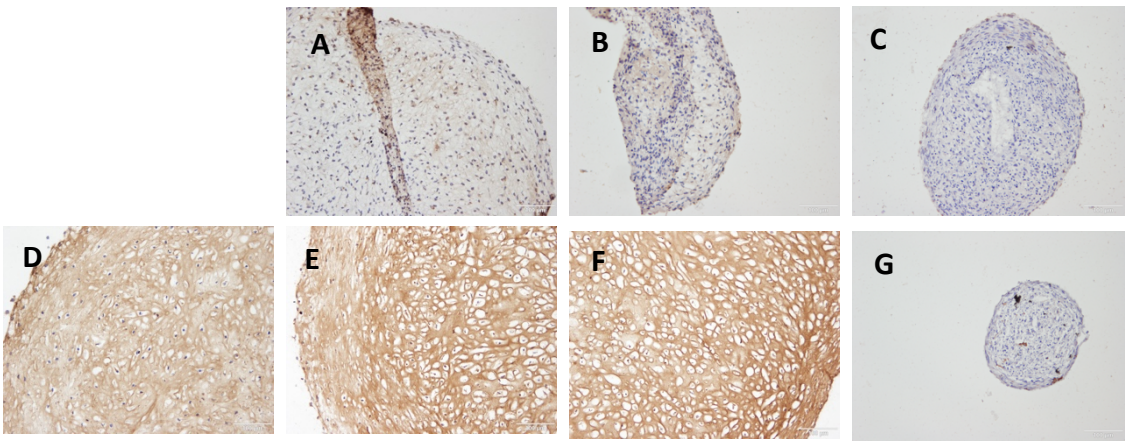


Figure 11. Collagen II staining at day 7 (A-C at 10x) and day 28 (D-G at 10x) of ACPCs pellets: AM4 donor (D), OA2 donor (B, E), AM2 donor (B, F),) and MSCs pellets (C, G), all cultured with one shot of BMP9 in the differentiation medium.

Immunohistological analyses for type I collagen were also conducted, since the existence of this protein in samples indicates the presence of fibrocartilage. With BMP9 in the chondrogenic differentiation medium, MSCs sample at day 7 (Fig. 12 D) showed a strong positivity for this protein in one of the laterals of the pellet, while the three ACPCs donors barely did (Fig. 12 A-C). At the last day of chondrogenic differentiation, all ACPCs donors showed a vague Col-I positivity, especially in the pellets external part (Fig. 12 E-G).

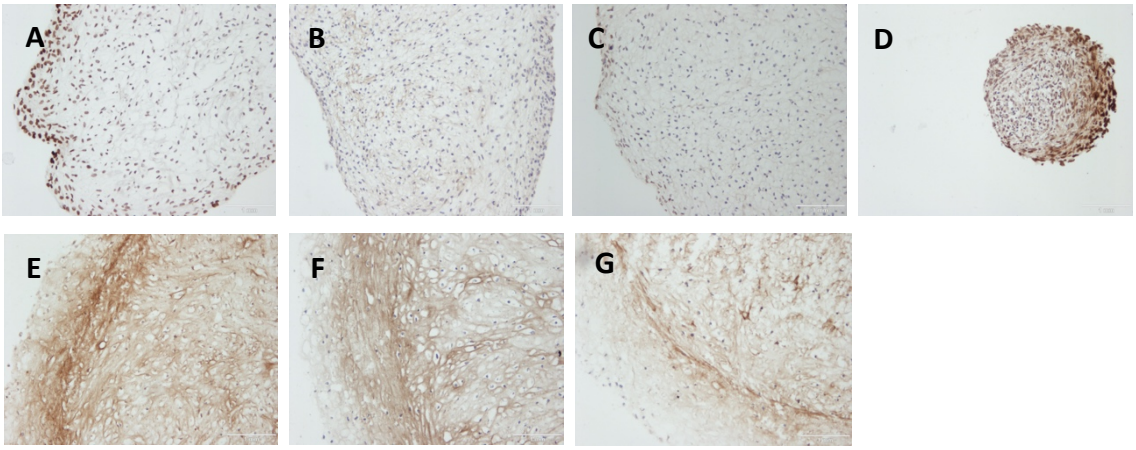


Figure 12. Collagen I staining at day 7 (A-D at 10x) and day 28 (E-G at 10x) of ACPCs pellets: AM4 donor (A-D), OA2 donor (B, E), AM2 donor (C, F),) and MSCs pellets (D), all cultured with BMP9 in the differentiation medium.

Samples cultured with TGFβ1 showed a more defined Col-I presence at day 7 of culture covering the pellets perimeter (Fig. 13 A-C). After 28 days of culture, a strong collagen type I signal could be detected in all the three ACPCs donors (Fig. 13 D-F), notably in the first donor AM4. MSCs pellet was also partly positive stained (Fig. 13 G)

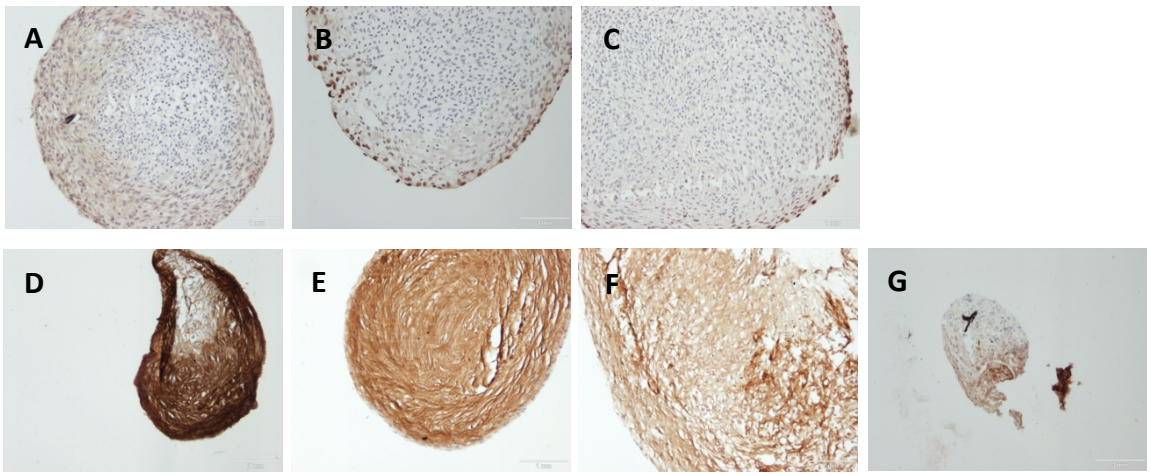


Figure 13. Collagen I staining at day 7 (A-D at 10x) and day 28 (E-G at 10x) of ACPCs pellets: AM4 donor (A-D), OA2 donor (B, E), AM2 donor (C, F),) and MSCs pellets (D), all cultured with TGFβ1 in the differentiation medium.

Finally, the samples cultured with one shot of BMP9 the first day of culture, showed at day 7 a modest positive signal for Col-I (Fig. 14 A-B), however the signal increased after 28 days of culture, especially in the first donor of ACPCs AM4 (Fig. 14 C), where we observed an homogeneous expression of Col-I, whereas in the other two donors the expression of this protein was localized in the external parts of the pellet (Fig. 14 D, E).

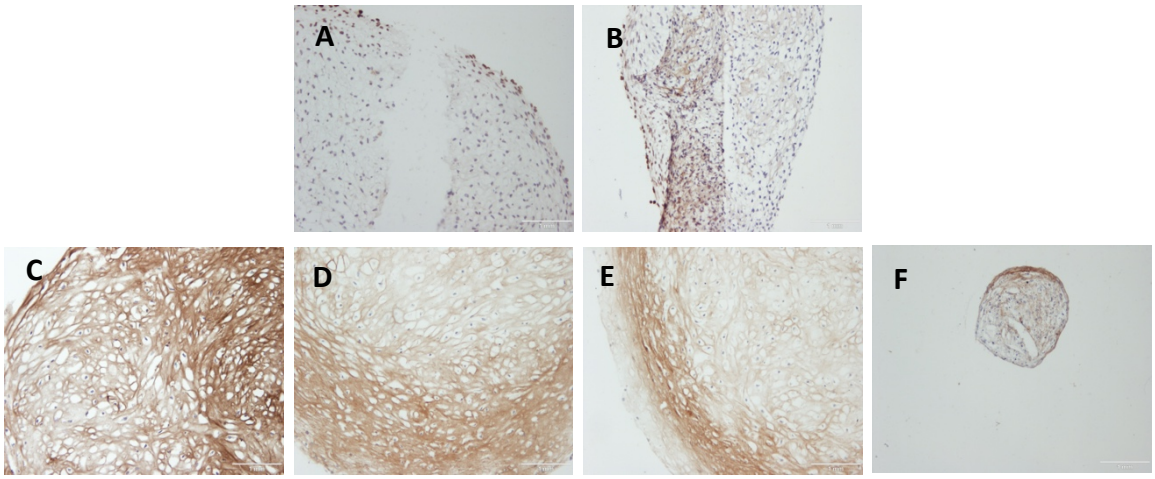


Figure 14. Collagen I staining at day 7 (A-B at 10x) and day 28 (C-F at 10x) of ACPCs pellets: AM4 donor (C), OA2 donor (A, D), AM2 donor (B, E),) and MSCs pellets (F), all cultured with one shot of BMP9 in the differentiation medium.

Immunofluorescence: collagen II and collagen VI

In order to detect collagen type II, a major component in the ECM of the articular cartilage, and collagen type VI, a pericellular protein also present in this type of cartilage, immunofluorescence detection assays were performed on pellets at day 28 of culture. In AM4 donor (Fig. 15), under the BMP9 (Fig. 15 A) and one shot of BMP9 (Fig. 15 C) conditions we observed a positive signal for Col-II, while under TGFβ1 conditions (Fig. 15 B) there was no signal of it, and we just appreciated the cell nuclei and actin signals. Col-VI signal was stronger in one shot of BMP9 medium (Fig. 15 D), however, we detected a slighter signal of it in BMP9 medium as well (F).

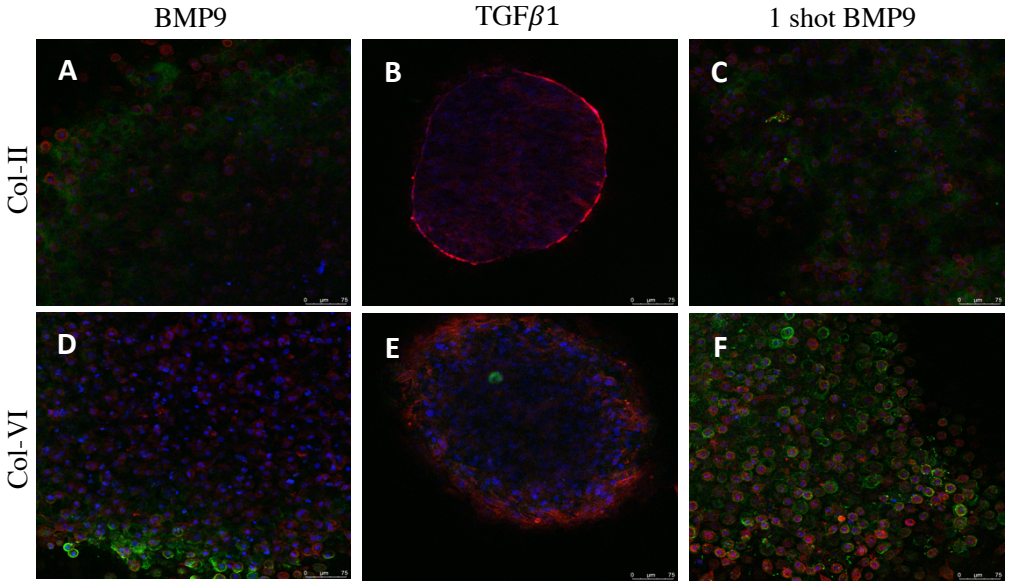


Figure 15. Collagen II, IV immunofluorescence staining of ACPCs pellets (donor AM4), at day 28 of culture at 20x. Under BMP9 (A, D), TGFβ1 (B, E) and one shot of BMP9 (C, F) conditions. DAPI (blue): nuclei, TRITC (red): actin, ALEXA 680 (green) col-II or VI.

We obtained similar results with the second ACPCs donor assessed OA2 (Fig. 16). Coll-II signal was detected homogeneously under BMP9 (Fig. 16 A) and one shot BMP9 (Fig. 16 C) conditions. There was no signal of this protein under TGFβ1 (Fig. 16 B) conditions as previously observed in AM4 donor. The signal of Col-VI was expressed around some cells of the sample under BMP9 (Fig. 16 D) and one shot of BM9 (Fig. 16 F) conditions, and also slightly detected in the pellet cultured with TGFβ1 (Fig. 16 E).

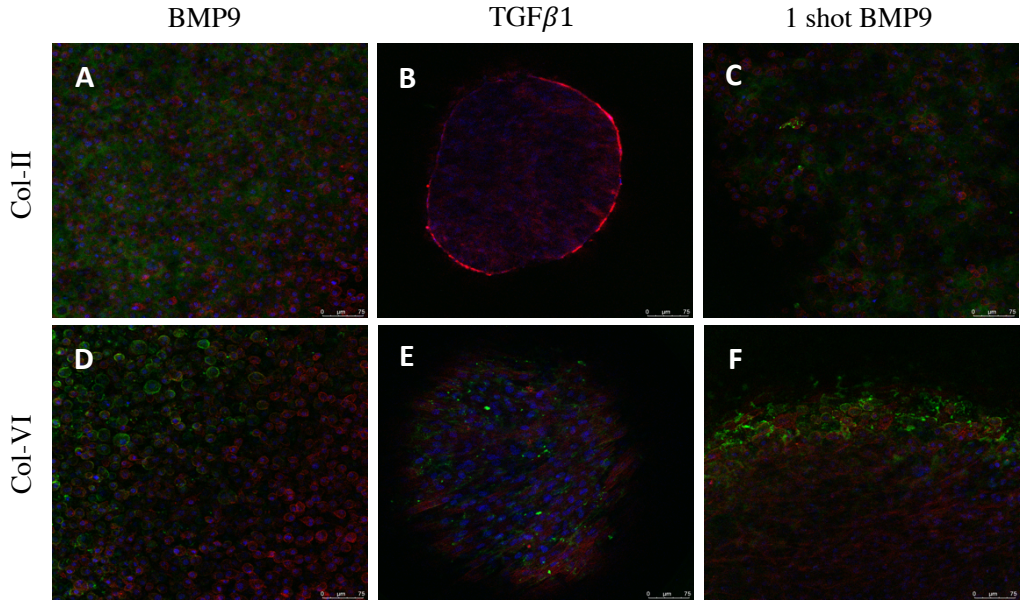


Figure 16. Collagen II, IV immunofluorescence staining of ACPCs pellets (donor OA2), at day 28 of culture at 20X. Under BMP9 (A, D), TGFβ1 (B, E) and one shot of BMP9 (C, F) conditions. DAPI (blue): nuclei, TRITC (red): actin, ALEXA 680 (green) col-II or VI.

Finally, the last donor, AM2 (Fig. 17), was also assessed. In this case, the protein signals were similar to previously exposed donors. Col-II stained positively for BMP9 (Fig. 17 A) and one shot of BMP9 (Fig. 17 C) conditions, and it was not stained in TGFβ1 medium (Fig. 17 C). Col-VI signal was observed pericellularly in both mediums containing BMP9 (Fig. 17 D, F), and was less expressed in the pellet cultured in TGFβ1 (Fig. 17 E) conditions.

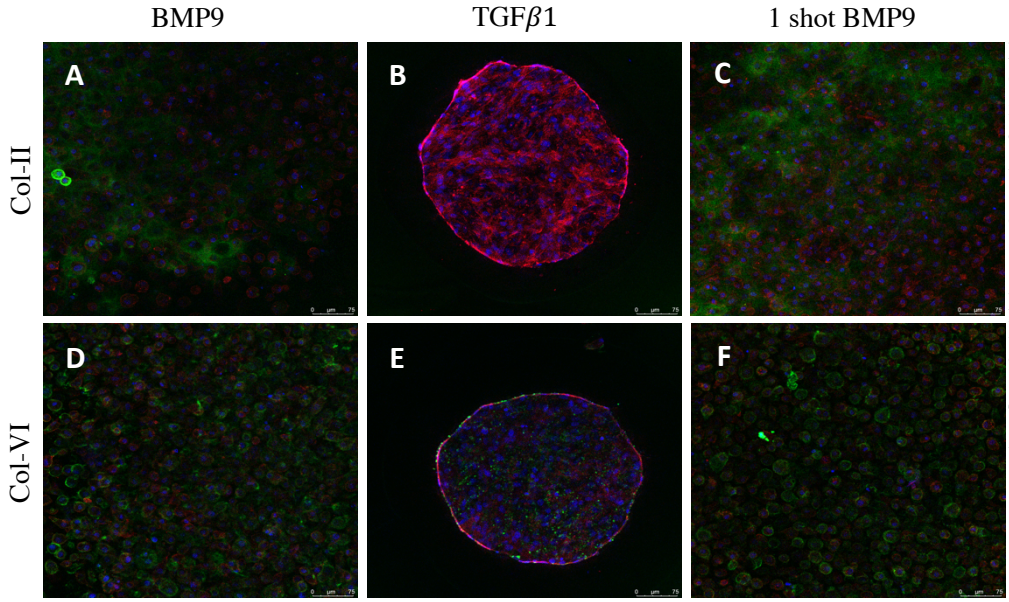


Figure 17. Collagen II, IV immunofluorescence staining of ACPCs pellets (donor AM2), at day 28 of culture at 20X. Under BMP9 (A, D), TGFβ1 (B, E) and one shot of BMP9 (C, F) conditions. DAPI (blue): nuclei, TRITC (red): actin, ALEXA 680 (green) col-II or VI.

Gene expression: quantitative-PCR

We analyzed the gene expression for Collagen type X, Notch1, Cadherin , RunX2 and Sox 9 in our different ACPCs donors and MSCs samples after day 1 and day 7 of culture. The obtained results contained a lot of errors, that might be due to a dimerization of the primers in the q-PCR master mix or an error in the protocol. The given mistakes could also come from a lack of expression of the genes, which would not count as errors. Col-X expression (Fig. 18 A), which indicates endochondral formation, could only be detected at day 1 in the MSCs samples cultured under TGFβ1 condition. Notch1 expression (Fig. 18 B), which indicates stem cell-like properties, was not detectable at day 1, however, it was detectable at day 7 in the ACPCs donor OA2. Cadherin expression (Fig. 18 C) was observed at day 1 in the MSCs samples under TGFβ1 and one shot of BMP9 conditions, and this decreased significantly at day 7. RunX2 expression (Fig. 18 D) that indicates the chondrocytes terminal differentiation, was detected at day 1 in AM4/OA2 under BMP9 conditions, and MSCs under TGFβ1 conditions, after 7 of cultured the expression of RunX2 was just observed in MSCs samples. Finally, Sox9 expression (Fig. 18 E), which is another stem cell marker, was only present in AM4 donor after 7 days of culture.

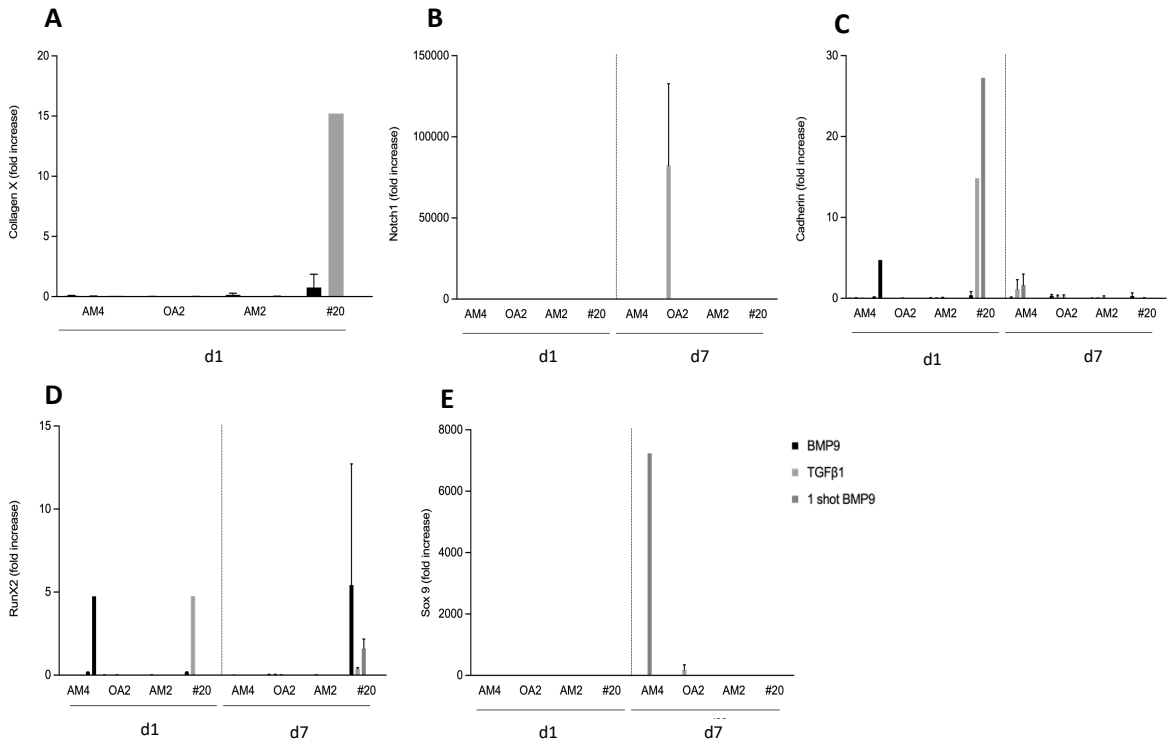


Figure 18. q-PCR analysis of ACPCs (donors AM4, OA2, AM2) and MSCs (#20) pellets at day 1 and day 7. Relative gene expression of Collagen X (A), Notcht1 (B), Cadherin (C), RunX2 (D), Sox9 (E), normalized against the housekeeping gene HPRT1.

3.4. Encapsulation of ACPCs in NorHa N-Cadherin mimetic hydrogels

Biochemical analysis: Picogreen, DMMB

DNA quantification (Fig. 19 A) showed that the number of cells after chondrogenic differentiation increased significantly in both NorHa hydrogels with and without peptide, independently from the medium condition. In terms of neocartilage ECM deposition (Fig. 19 B), we observed that, in both type of hydrogels, after 28 days of culture, there was an increment of GAGs deposition when samples were cultured with BMP9. The increment in GAGs/DNA (Fig. 19 C) was significantly higher after 28 days of culture in both hydrogels when these were cultured with BMP9, however, there were no significant differences between the two types of hydrogels.

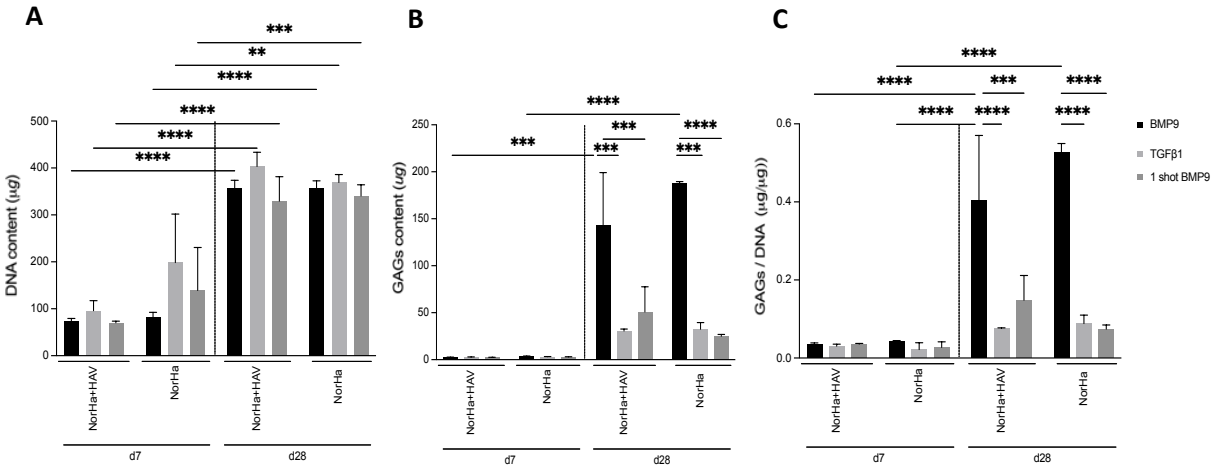


Figure 19. Quantification of DNA (A) and GAGs (B) in NorHa+HAV and NorHA hydrogels encapsulated with ACPCs after 7 and 28 days of culture. GAGs quantification was normalized to DNA content (C) after 7 and 28 days of culture. Significant difference between columns is shown below each end of lines (* means $p < 0.0332$, ** means $p < 0.0021$, *** means $p < 0.0002$, **** means $p < 0.0001$). The graph shows the mean (M) with the standard deviation (SD) for each group (n=3).

Histological assessment: Safranin-O

In order to analyze the formation of new articular cartilage in the encapsulated hydrogels after the chondrogenic differentiation, a qualitative analysis of GAGs was performed. Before start analyzing the obtained results, we had to take into account the results obtained with the hydrogel control without any cells encapsulated inside (Fig. 22). This hydrogel resulted positive (pink) for GAGs formation, which meant that probably one of the components of the structure also got stained with the Safranin O assay, giving a false positive. Based on this, we had to analyze the observed results with caution, and choosing another method to detect this polysaccharide would have been recommended. After 7 days of culture (Fig. 20 A-C, Fig. 21 A-C), we observed similar types of signals for both, NorHa+HAV and NorHa hydrogels, independently of the growth factor used in each group, this signal is pinkish and very similar to the one obtained in the control. However, after 28 days of culture, in both types of hydrogels, the samples cultured with BMP9

(Fig. 20 D and Fig. 21 D), appeared to have a darker pink/reddish meaning a possible increment in the ECM formation. The NorHa+HAV samples cultured with TGFβ1 (Fig. 20 E) showed an interesting staining, with a positive signal for GAGs and a blue band that represents the fibrocartilaginous part also present in the real tissue.

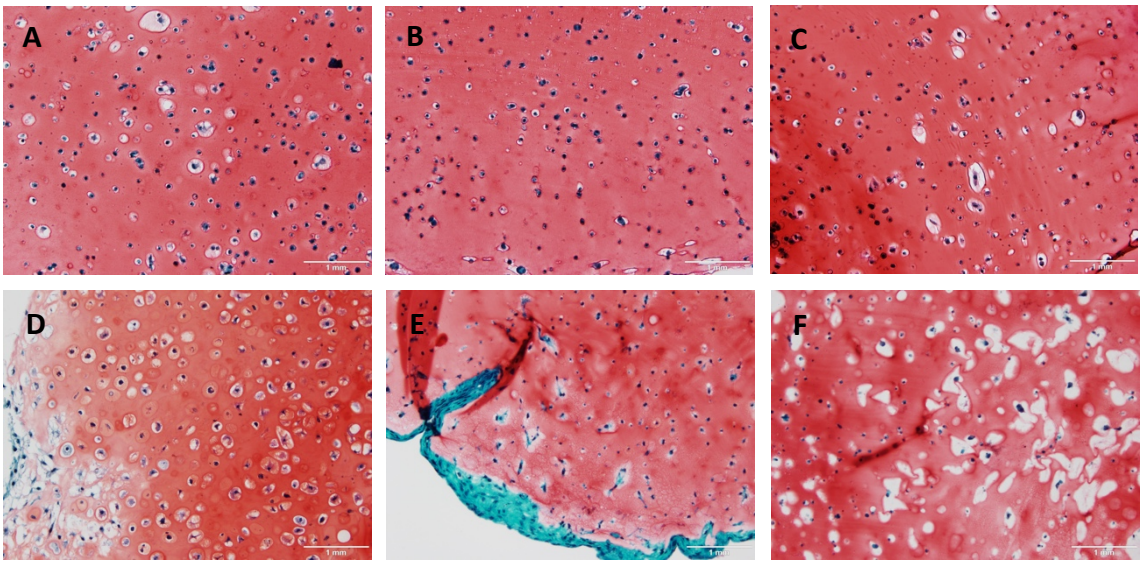


Figure 20. Safranine O staining at day 7 (A-C at 10x) and day 28 (D-F at 10x) of encapsulated ACPCs in NorHA+HAV hydrogels. The chondrogenic differentiation medium contained BMP9 in A and D, TGFβ1 in B and E, and one shot of BMP9 the first day of culture in C and F.

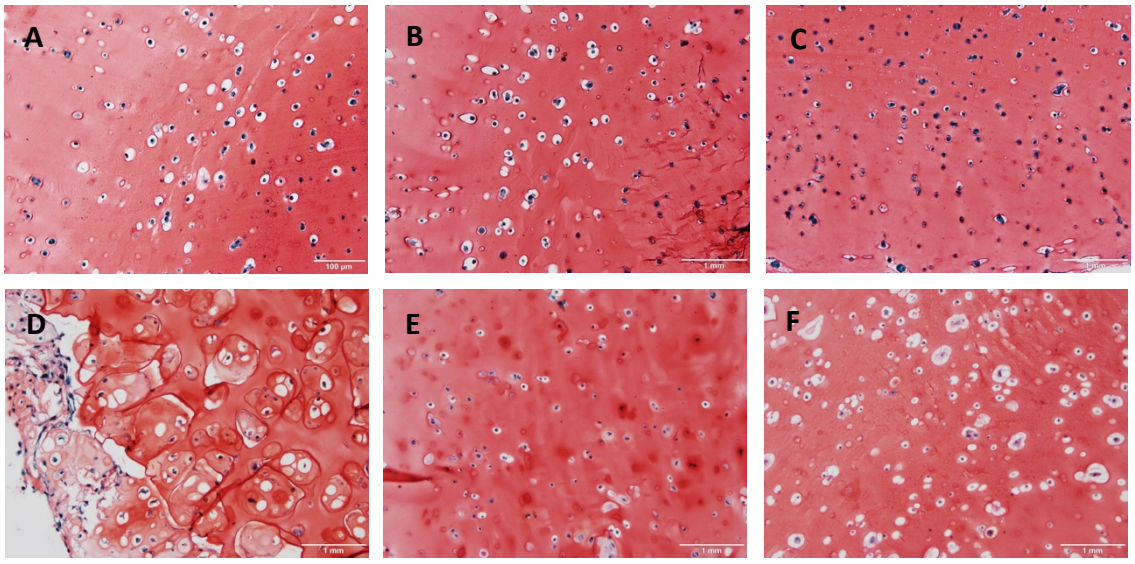


Figure 21. Safranine O staining at day 7 (A-C at 10x) and day 28 (D-F at 10x) of encapsulated ACPCs in NorHA hydrogels. The chondrogenic differentiation medium contained BMP9 in A and D, TGFβ1 in B and E, and one shot of BMP9 the first day of culture in C and F.

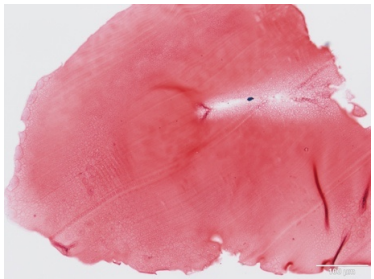


Figure 22. Safranine O staining of control hydrogel: NorHa+HAV without encapsulated cells.

Histological assessment: Collagen-II and Collagen-I

Immunohistological analyses of type II collagen were performed to detect neocartilage ECM formation in our hydrogels. We observed very similar staining for Col-II in both types of hydrogels, with and without N-cadherin mimetic peptide. At day 7 (Fig. 23 A-C and Fig. 24 A-C), there was barely any signal of Col-II in any of the samples. Col-II positivity was detected in NorHa+HAV (Fig. 23 D) and NorHa (Fig. 24 D) cultured with BMP9 after 28 days. NorHa+HAV hydrogels showed an homogeneous distribution of the ECM synthesis, while in NorHa hydrogels the signal was lower and had a rounded/isolated display.

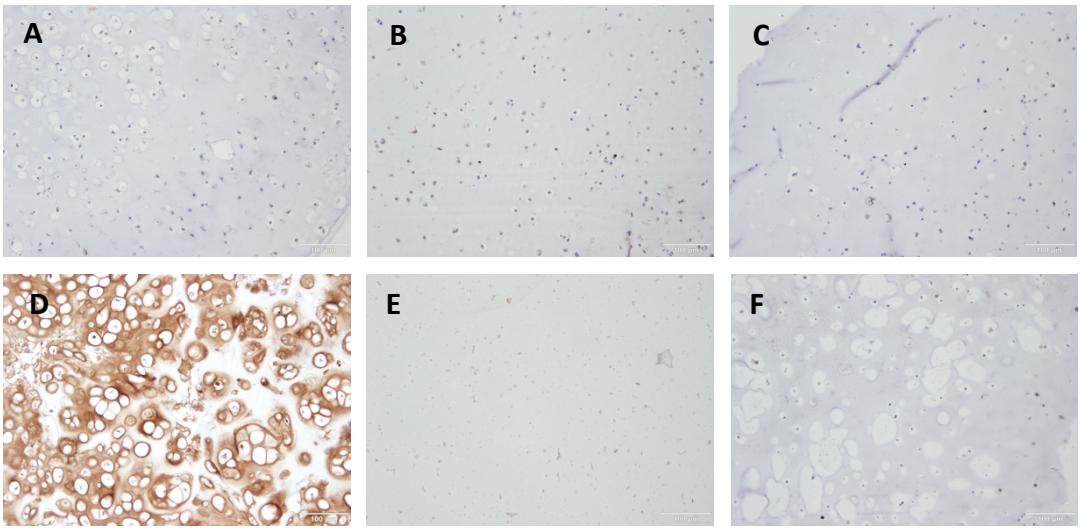


Figure 23. Collagen II staining at day 7 (A-C at 10x) and day 28 (D-F at 10x) of encapsulated ACPCs in NorHa+HAV hydrogels. The chondrogenic differentiation medium contained BMP9 (A, D), TGFβ1 (B, E), one shot of BMP9 the first day of culture (C, F).

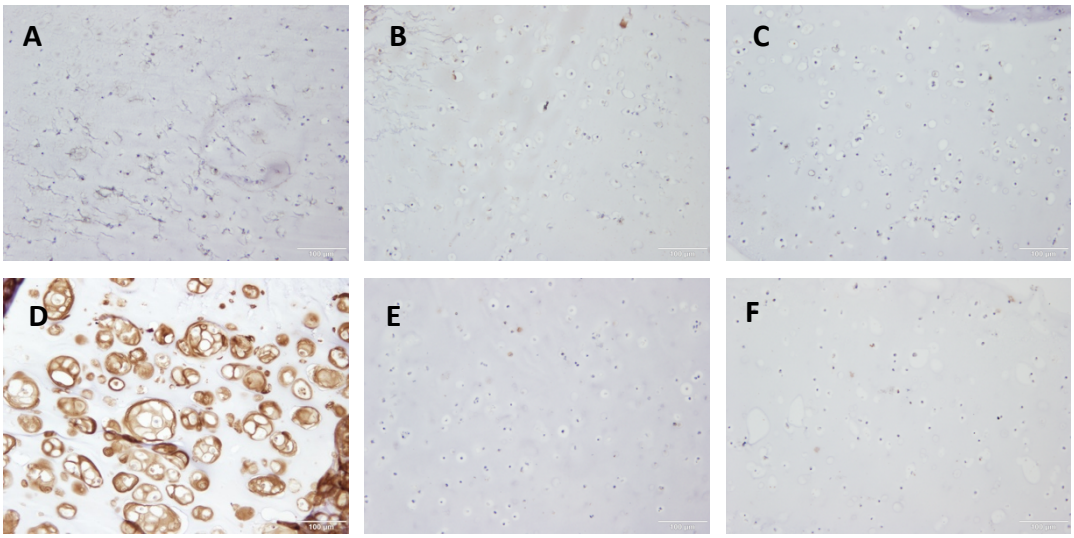


Figure 24. Collagen II staining at day 7 (A-C at 10x) and day 28 (D-F at 10x) of encapsulated ACPCs in NorHA hydrogels. The chondrogenic differentiation medium contained BMP9 (A, D), TGFβ1 (B, E), and one shot of BMP9 the first day of culture (C, F).

In order to detect the presence of fibrocartilage, immunohistological assays for collagen type I were also performed. For both types of hydrogels we observed a slight positive signal for this protein already at day 7 of culture, especially, under the BMP9 and TGFβ1 conditions (Fig. 25 A, B and Fig. 26 A, B). Nevertheless, the positive signal increased significantly after 28 days of culture. In the BMP9 condition, NorHa+HAV hydrogel (Fig. 25 D), showed an homogeneous expression of Col-I, while in NorHa hydrogel (Fig. 26 D) the expression appeared mainly in the surroundings. Under TGFβ1 effect, NorhHa+HAV hydrogel (Fig. 25 E), had a higher expression of Col-I, stronger in the external part and not defined towards the center of the hydrogel. The expression of this protein in both hydrogels cultured with one shot of BMP9 was low (Fig. 25 F, Fig. 26 F).

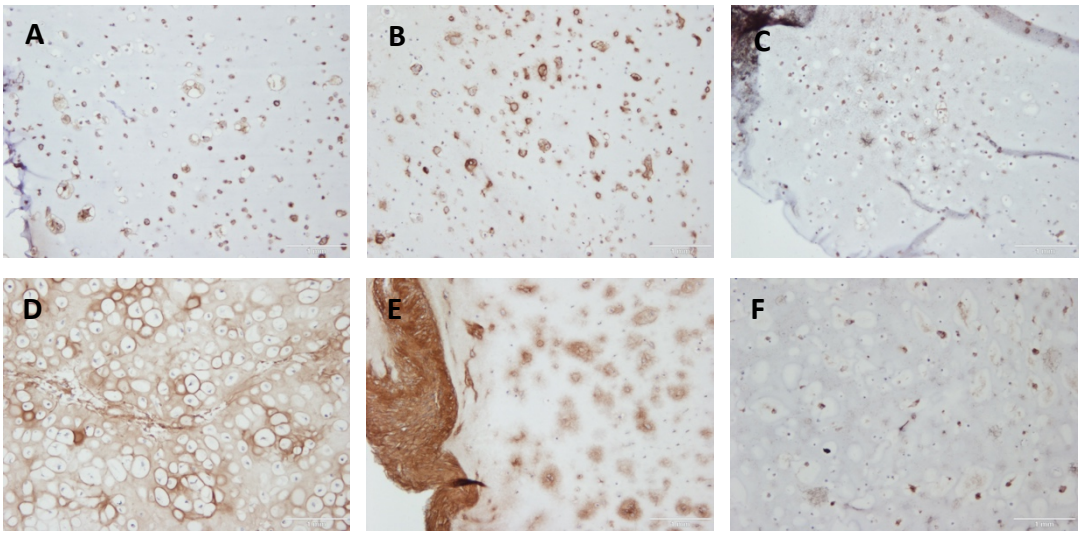


Figure 25. Collagen I staining at day 7 (A-C at 10x) and day 28 (D-F at 10x) of encapsulated ACPCs in NorHa+HAV hydrogels. The chondrogenic differentiation medium contained BMP9 (A, D), TGFβ1 (B, E), one shot of BMP9 the first day of culture (C, F).

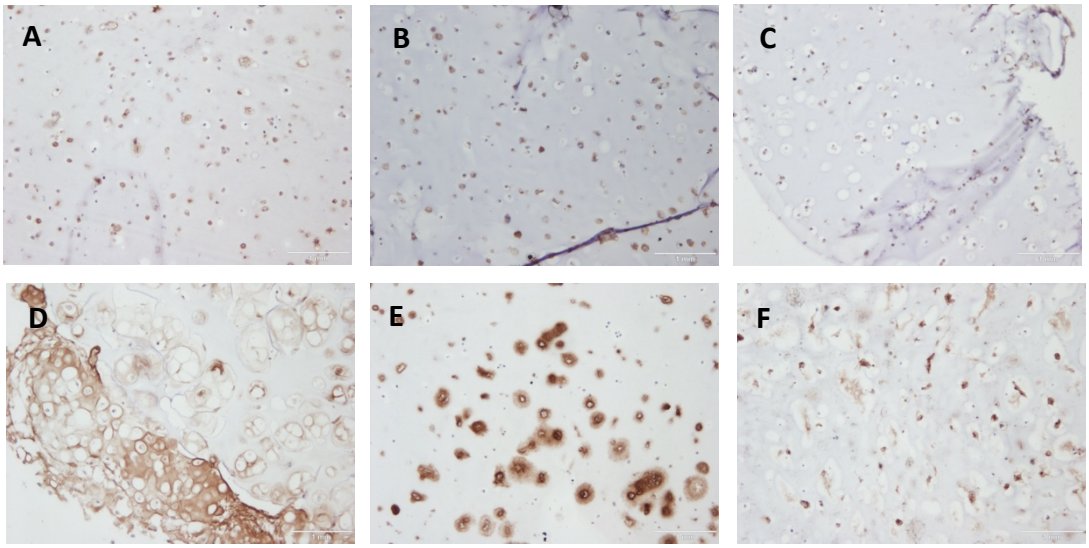


Figure 26. Collagen I staining at day 7 (A-C at 10x) and day 28 (D-F at 10x) of encapsulated ACPCs in NorHA hydrogels. The chondrogenic differentiation medium contained BMP9 (A, D), TGFβ1 (B, E), one shot of BMP9 the first day of culture (C, F).

Immunofluorescence: collagen II and collagen VI

Aiming to detect collagen type II and collagen type VI in our hydrogels after chondrogenic differentiation of the encapsulated ACPCs, we performed an immunofluorescence staining at day 28. Pictures were taken in order to detect single cells inside the hydrogel, however, there was a considerable amount of cell clusters that also appear in the images. On the one hand, NorHa+HAV samples (Fig. 27) stained positively for Col-II when cultured under BMP9 (Fig. 27 A, C) conditions. The medium containing just one shot of BMP9 (Fig. 27 C) had a vague positive signal as well. The hydrogel cultured in TGFβ1 medium (Fig. 27 B), did not express any positive signal for this protein. We observed a very low Col-VI protein expression, being just significant the expression in the sample cultured with BMP9 (Fig. 27 D). On the other hand, NorHa hydrogels (Fig. 28) did not show any positivity for Col-II (Fig. 28 A-C), nevertheless Col-VI signal was detected in the mediums containing BMP9 (Fig. 28 D, F) surrounding cells and cell clusters.

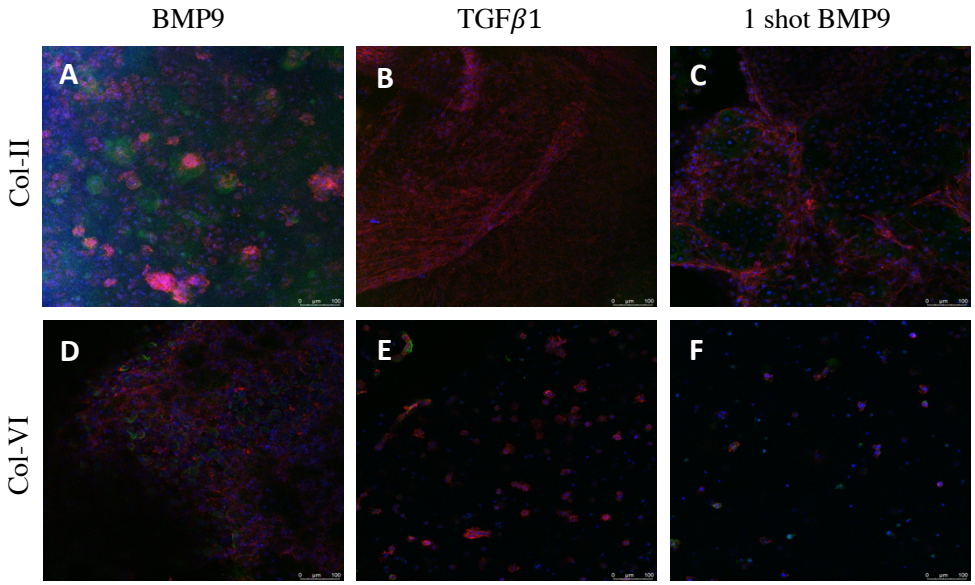


Figure 27. Collagen II, IV immunofluorescence staining of NorHa+HAV hydrogels encapsulated with ACPCs at day 28 of culture at 20X. Under BMP9 (A, D), TGFβ1 (B, E) and one shot of BMP9 (C, F) conditions. DAPI (blue): nuclei, TRITC (red): actin, ALEXA 680 (green) col-II or VI.

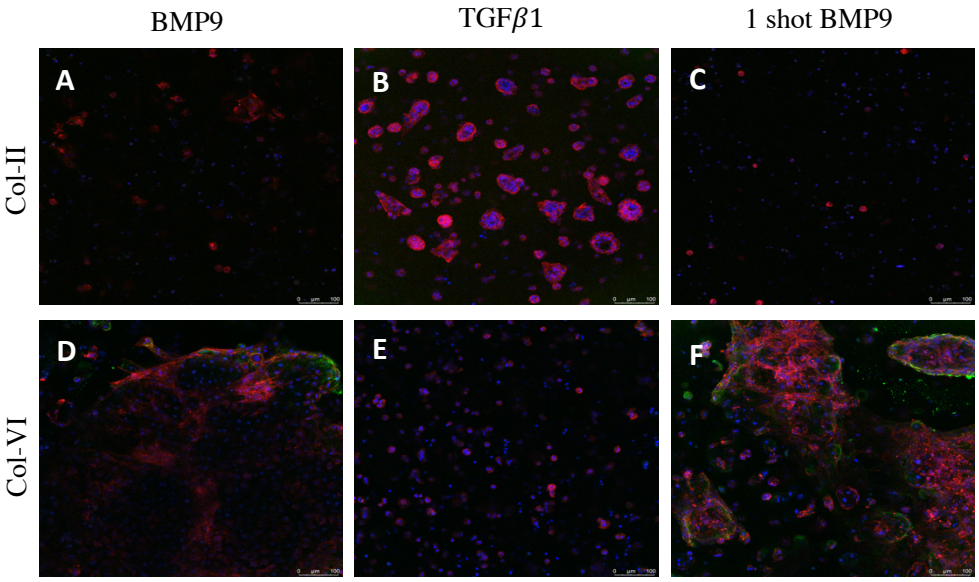


Figure 28. Collagen II, IV immunofluorescence staining of NorHa hydrogels encapsulated with ACPCs at day 28 of culture at 20X. Under BMP9 (A, D), TGFβ1 (B, E) and one shot of BMP9 (C, F) conditions. DAPI (blue): nuclei, TRITC (red): actin, ALEXA 680 (green) col-II or VI.

3.5. Cell-laden (ACPCs) PCL meshes

Biochemical analysis: Picogreen, DMMB

After 28 days, the amount of DNA content (Fig. 29 A) decreased in a non-significant manner in both types of models, cell-laden meshes and ACPCs pellets, for both conditions, BMP9 and one shot of BMP9. In terms of neocartilage ECM deposition (Fig. 29 B), the increment of GAGs content was significantly higher in the mesh structures cultured with BMP9 and the only cell samples cultured with one shot of BMP9 the first day of culture. Samples expressed not significant results of GAGs normalized to DNA content in any of the structures or conditions used (Fig. 29 C).

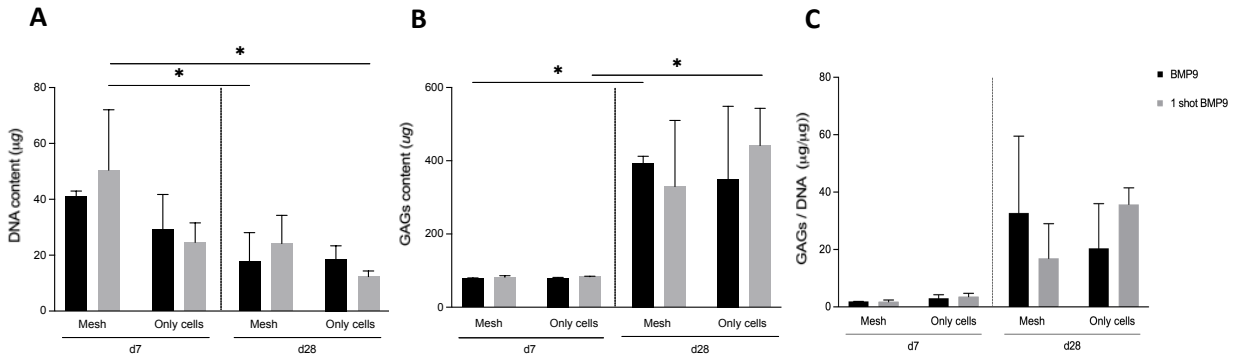


Figure 29. Quantification of DNA (A) and GAGs (B) in ACPCs-laden PCL meshes and ACPCs rounded pellets, GAGs quantification was normalized to DNA content (C) after 7 and 28 days of culture. Significant difference between columns is shown below each end of lines (* means $p < 0.0332$, ** means $p < 0.0021$, *** means $p < 0.0002$, **** means $p < 0.0001$). The graph shows the mean (M) with the standard deviation (SD) for each group (n=3).

Mechanical testing: DMA

The mechanical properties of the cell-laden PCL meshes and ACPCs rounded pellets are shown on Figure 30. The compression modulus of both culture systems was measured after 7 and 28 days of culture. After 7 days of culture, the rounded cells pellet showed higher compression modulus values compared to the cell-laden mesh structure, under the one shot of BMP9 condition in the first day of culture. After 28 days of chondrogenic differentiation, we observed that cell-laden meshes had a higher compression modulus compared to the ACPCs pellets when cultured with one shot of BMP9, whereas, there was not a significant difference in stiffness at day 28 in between cell-laden meshes and only cells structures when cultured with BMP9, moreover, these had lower compression moduli values.

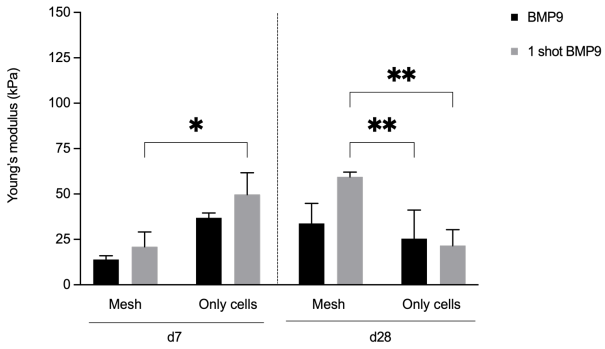


Figure 30. Quantification of DNA (A) and GAGs (B) in ACPCs-laden PCL meshes and ACPCs rounded pellets after 7 and 28 days of culture. Significant difference between columns is shown below each end of lines (* means $p < 0.0332$, ** means $p < 0.0021$, *** means $p < 0.0002$, **** means $p < 0.0001$). The graph shows the mean (M) with the standard deviation (SD) for each group (n=3).

Immunofluorescence: collagen II and collagen VI

In order to detect the presence of collagen type II and collagen type VI, we performed an immunofluorescence assay in our samples. At day 7, cell-laden PCL meshes (Fig. 31) samples tested positive mainly for Col-VI, in both conditions containing BMP9. Nevertheless, the aspect of the protein distribution seemed different for each condition, while in the continuously refreshed medium with BMP9 (Fig. 31 C), Col-VI was following the mesh pattern, in the one shot of BMP9 (Fig. 31 D), the protein surrounds the ACPCs. Col-II had a slight signal following the mesh structure in the BMP9 (Fig. 31 A) condition, however, the signal was nonexistent in the one shot media (Fig. 31 B). At day 7, samples containing only cells (Fig. 32) tested negative for Col-II signal in both conditions (Fig. 32 A, B). Col-VI was only observed in the one shot of BMP9 medium (Fig. 32 D). After 28 days of chondrogenic differentiation culture, in cell-laden PCL meshes (Fig. 33) we observed a small vague positive signal for Col-II in the BMP9 medium (Fig. 33 A), oppositely to the one shot of BMP9 (Fig. 33 B) medium, were there was no signal. Col-VI signal was present in both types of BMP9 medium (Fig. 33 C, D). In the only ACPCs structures (Fig. 34), Col-II was not found, and Col-VI positivity was observed only in the media containing BMP9 during the whole differentiation process (Fig. 34 B).

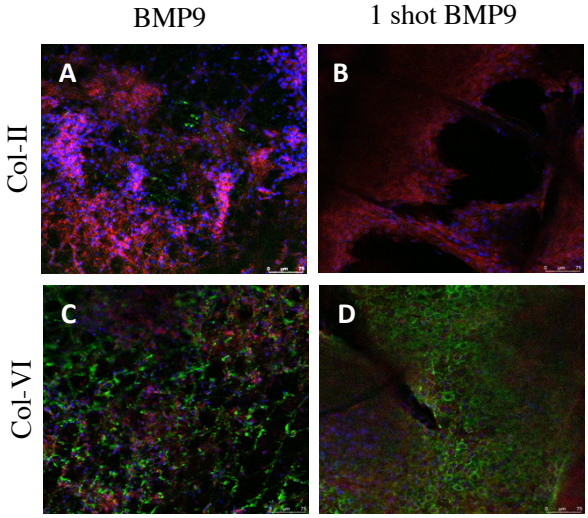


Figure 31. Collagen-II, VI immunofluorescence detection in ACPCs-laden PCL meshes at day 7 of culture. Under BMP9 (A,C) and one shot of BMP9 (B, D) medium.

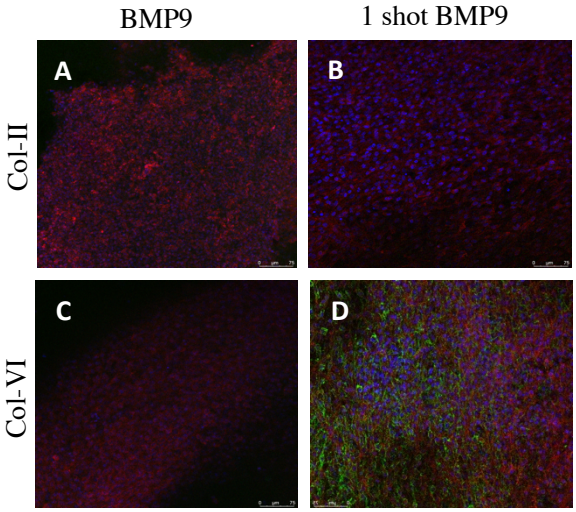


Figure 32. Collagen-II, VI immunofluorescence detection in ACPCs rounded pellets at day 7 of culture. Under BMP9 (A,C) and one shot of BMP9 (B, D) medium.

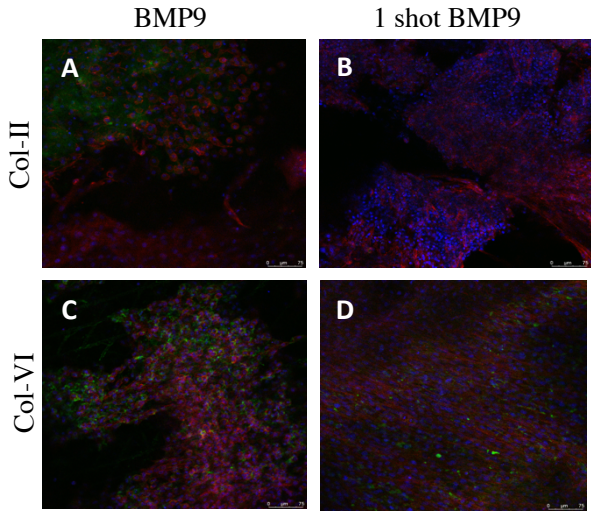


Figure 33. Collagen-II, VI immunofluorescence detection in ACPCs-laden PCL meshes at day 28 of culture. Under BMP9 (A,C) and one shot of BMP9 (B, D) medium.

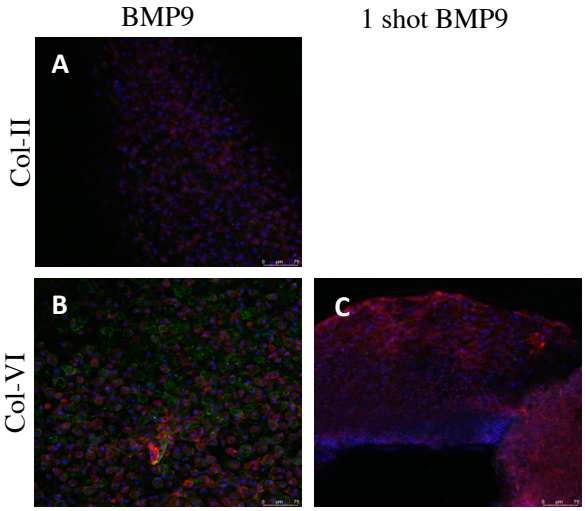


Figure 34. Collagen-II, VI immunofluorescence detection in ACPCs rounded pellets at day 28 of culture. Under BMP9 (A,C) and one shot of BMP9 (B, D) medium.

4. DISCUSSION

4.1. ALK-1 as the receptor of BMP9 in human ACPCs

In order to comprehend the BMP9 chondrogenic boosting effects on ACPCs, in this study we first focused on its cell targets and receptors. Previous studies have demonstrated that Alk-1 is the principal receptor of this growth factor in endothelial cells.[31] This binding however, was still not proven in ACPCs. For this reason, in this project we blocked this receptor in ACPCs and MSCs, with a specific small molecule inhibitor[32], to prove if ALK-1 is the receptor that allows the entrance of BMP9 signal inside the cell, and subsequently, that produce the Smad 1/5/8 cascade phosphorylation. On the one hand, here we have shown that after blocking Alk-1 in ACPCs during the differentiation process, chondrogenic features were not expressed under BMP9 conditions, whereas in the non-blocked samples, BMP9 was the condition with the best chondrogenic differentiation results. These results proved that Alk-1 is the principal receptor of this growth factor in ACPCs. On the other hand, MSCs results suggested that BMP9 has a lower chondrogenic effect in their differentiation process, indicating that MSCs and ACPCs might have different amounts and type of ALK cell receptors that would justify their different responses towards BMP9 in the culture medium. We could relate the results in Finnson et al.[33], where they demonstrated that TGFβ1 also acts through Alk-1 in human chondrocytes and that this binding decreases the TGFβ1 /Alk-5 binding effects, to our results. When Alk-1 was blocked in MSCs, TGFβ1 showed the best chondrogenic conditions in these cells, indicating that this growth factor might be able to work without being suppressed by other bindings such as TGFβ1/Alk-1. It should be also noted that the synergy condition without dexamethasone (containing TGFβ1 and BMP9) also produced interesting results in terms of GAGs production in ACPCs, leading to a possible interaction between these two factors in the native tissue for a better, more controlled and realistic differentiation. Since the relation between these two growth factors has been already studied in endothelial cells and chondrocytes, further investigation should be conducted in the chondrogenic differentiation of ACPCs.

4.2. ALK-1 and ALK-5 expression in ACPCs, MSCs and Chondrocytes

To further understand the response of the cells towards TGFβ1 and BMP9, we investigated the protein expression, through Western Blot, of the cell receptors Alk-1 and Alk-5 in three different cells: ACPCs, MSCs and Chondrocytes. These three types of cells have been considered suitable candidates for articular cartilage cell therapy, and since their response to the same growth factors varies, here, we studied possible cellular differences between cells that could explain their differential behavior.

Our results showed the highest expression for Alk-1 in chondrocytes, followed by a lower expression in ACPCs and MSCs. However, we obtained opposite results for the Alk-5 expression, being higher in MSCs and lower in ACPCs and MSCs. Unfortunately the results were not able to be normalized to a positive control, and subsequently, the protein amount quantification was not performed. Nevertheless, the blot results showed differential expression for both cell receptors, thus maybe explaining the different reactions of the cells to each growth factor. On the one hand, a higher expression of Alk-1 in ACPCs would explain their increased growth and differentiation when cultured under BMP9 conditions. On the other hand, the high expression of Alk-5 in MSCs would also explain their better differentiation into chondrocytes when cultured in mediums containing TGFβ1, as Alk-5 is the principal receptor of it. The relation between Alk-1 and Alk-5 has been previously questioned, some studies proposed that these two receptors have opposite functions in endothelial cells [34]. Another study proposed that TGFβ/Alk-5 binding inhibits BMP9 signaling in cartilage[20]. However, Shao et al.[35] demonstrated that the suppression of Alk-5 does not interfere in the BMP9 and Alk-1 interaction in endothelial cells. As previously mentioned, Finnson et al. proved that the binding TGFβ/Alk-1 attenuated the response of TGFβ/Alk-5 activation pathway in chondrocytes, thus decreasing the expression of cartilage specific proteins such as Col-II under the Smad 2/3 phosphorylation cascade. For these reasons and in accordance with our results, MSCs might express a higher amount of Alk-5 in order to differentiate, and chondrocytes, which are already differentiate, might express lower levels of Alk-5 and higher levels of Alk-1 to be able to maintain their phenotype, finding a balance in between synthesizing ECM and degrading it.[36] Hence, in order to find the best differentiation conditions for future cell therapies, more research about cellular receptors and their activation pathways would be recommended in human ACPCs.

4.3. BMP9 chondrogenic potential in three different ACPCs donors

To better comprehend the BMP9 enhancing effect on ACPCs, this was assessed on three different ACPCs donors. Moreover, in order to achieve the chondrogenic differentiation, the correct administration dose of BMP9 was also studied. Here we compared its effect by adding BMP9 during the whole differentiation period (28 days), by a one-time BMP9 addition on the first day of culture, or as a control, by adding TGFβ. There were no significant differences between the three ACPCs donors when cultured under both BMP9 conditions, suggesting that this growth factor had the same chondrogenic effect in all of them, and also meaning that the addition of one shot of BMP9 on the first culture day might be enough to start a controlled and long-lasting chondrogenic differentiation. Nevertheless, the Col-I positive signal after 28 days of culture, under both BMP9 conditions, might indicate the presence of fibrocartilage, which would not represent the formation of new articular cartilage. Thus, although this would not be convenient

when differentiating ACPCS into articular cartilage cells, the three donors expressed this signal also equally, meaning that ACPCs respond uniformly towards different cues. These results are relevant because, first, the chondrogenic potential of this growth factor might be applicable for all ACPCs, and second, a low dose of BMP9 might be sufficient to start the differentiation process in ACPCs pellet culture, thus, suggesting an adequate BMP9 dose to apply to other differentiation culture medias for ACPCs. Therefore, further studies with higher amount of ACPCs donors and starting low doses of BMP9 in the differentiation medium should be performed to confirm our findings.

4.4. ACPCs encapsulation in n-cadherin mimetic peptide NorHa hydrogels under BMP9 conditions

In the early embryonic development, cells transition from a cell-cell domain to an environment dominated by cell-ECM interactions. One of the principal objectives in the cartilage regeneration field is to create a structure that resembles cells native environment in order to help them to a faster and better differentiation into chondrocytes. As reported in Vega et al.[37], the addition of an N-cadherin mimetic peptide (HAV) inside of a MSCs-laden NorHa hydrogel increased the chondrogenic markers expression in these cells. For this reason, here we studied the effect of the mimetic peptide addition in NorHa hydrogels in combination with BMP9 and ACPCs. Our results suggested that, after 28 days of culture, the presence of the mimetic peptide did not increase the chondrogenic expression of ACPCs compared to the non-peptide hydrogels, thus, suggesting that these cells might not need this cue to evolve towards a chondrogenic phenotype. Recently, most of the research performed with these modified 3D hydrogels was done using MSCs. For instance, Bian et al.[30] demonstrated that N-cadherin mimetic peptides in hyaluronic acid hydrogels enhanced the MSCs chondrogenesis in *in vitro* and *in vivo* models. Furthermore, in Cosgrove et al.[27] it was proved that the addition of the mimetic peptide altered the stem cell perception of the extracellular microenvironment stiffness, thus generating a change in the downstream differentiation and proliferation cascades and modifying the fate commitment of the cells. Further studies should therefore be conducted with ACPCs in order to prove the possible effect of N-cadherin mimetic peptides in the chondrogenic differentiation of these cells. Finally, it must be noted that the enhancing chondrogenic effect of BMP9 tested positive for both types of hydrogels, meaning that its potential can be also applicable in 3D structures differentiation.

4.5. PCL meshes as a scaffold to create an ACPCs implant for articular cartilage

Lastly, in this study we assessed PCL rounded meshes as possible scaffolds to generate chondrogenic differentiated ACPCs implants for articular cartilage. We compared mesh and no mesh cell pellets under constant BMP9 and one-time BMP9 addition on the first day of culture.

Our results showed that by adding one shot of BMP9 and having the mesh reinforcement, ACPCs could differentiate and generate a structure with a high compression modulus, thus resembling one of the main properties of the native articular cartilage tissue. These results suggested that, whereas in pellet or hydrogel culture the constant addition of BMP9 was the best condition in terms of chondrogenic phenotype expression, here, a one-time addition of BMP9 might be sufficient to generate a suitable mesh implant with a controlled differentiation and proliferation of ACPCS. Therefore, further research must be done under these conditions to prove that ACPCs-laden PCL meshes, under a low dose of BMP9, might become a proper and functional articular cartilage implant.

5. LIMITATIONS AND FUTURE WORK

During this research project we have encountered different limitations and problems. Most of the time these were related to cell infections and experiment repetition, which led to a lack of replicates in some of the experiments. For future work we would therefore recommend a higher amount of replicates, especially for the cell-laden PCL mesh experiment. Moreover, q-PCRs analysis should be repeated for the three ACPCs donors and the NorHa+HAV hydrogels experiments, in order to tackle the faced problems such as possible protocol errors, late harvest days or primers polymerization. Furthermore, for the recognition of GAGs in the NorHA hydrogels, and due to a false positive signal in all our samples, we would suggest another technique to detect this element. Finally, Collagen type VI immunochemistry would have also been helpful in all of our samples to determine possible chondrogenic differentiation.

6. CONCLUSION

In this study we provided an overview of the BMP9 effects on human ACPCs in diverse differentiation culture systems. We confirmed that BMP9 binds and activates human ACPCs through the Alk-1 receptor. We also reported that BMP9 enhancing chondrogenic effect applies to different ACPCs donors, meaning that this effect can be generalized to all ACPCs. Furthermore, given the importance of the cell environment and scaffold in order to grow and differentiate cells into articular cartilage, two different 3D structures were tested in our project. The addition of N-cadherin mimetic peptides in Norbornene modified Hyaluronic Acid hydrogels did not improve the chondrogenic potential of ACPCs, however, groups containing BMP9 had the highest presence of chondrogenic signs, hence we concluded that BMP9 boosting effect can also be applied to ACPCs encapsulated in hydrogels. Finally, PCL meshes resulted in a potential cell-laden 3D structure to generate an articular cartilage implant for patients. Nevertheless, more research should be conducted to find optimal conditions to integrate the mesh in the native tissue, and to test the correct BMP9 administration dose. Overall, the results of this study contribute to further insight in the future of the articular cartilage regeneration field.

7. ACKOWLEDGEMENTS

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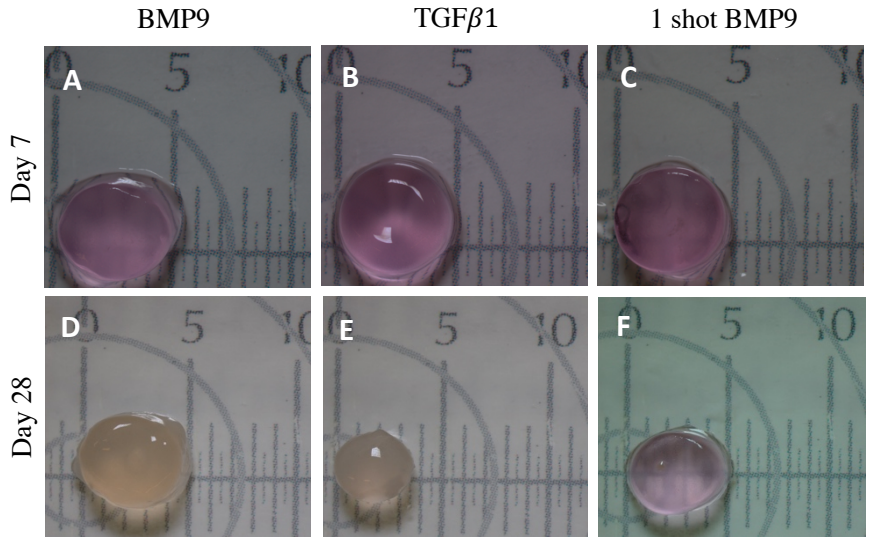
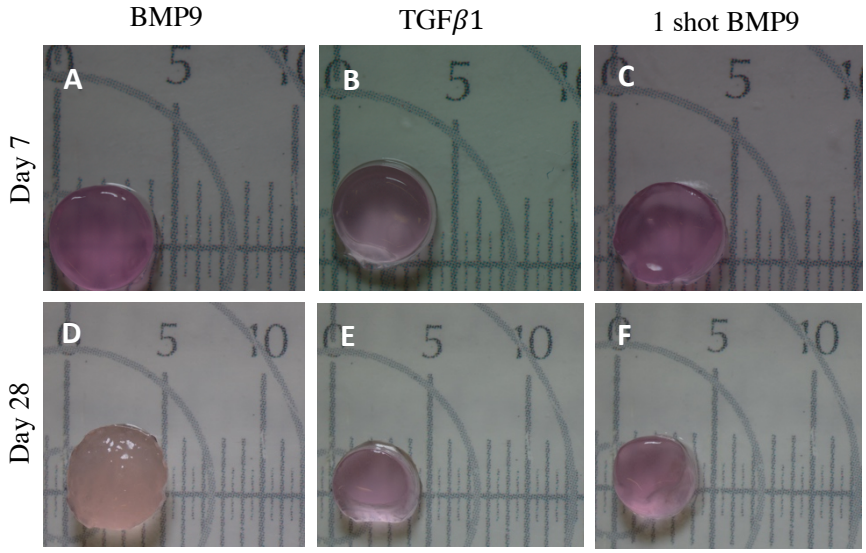
9. SUPLEMENTARY FIGURES

Encapsulation of ACPCs in NorHa N-Cadherin mimetic hydrogels

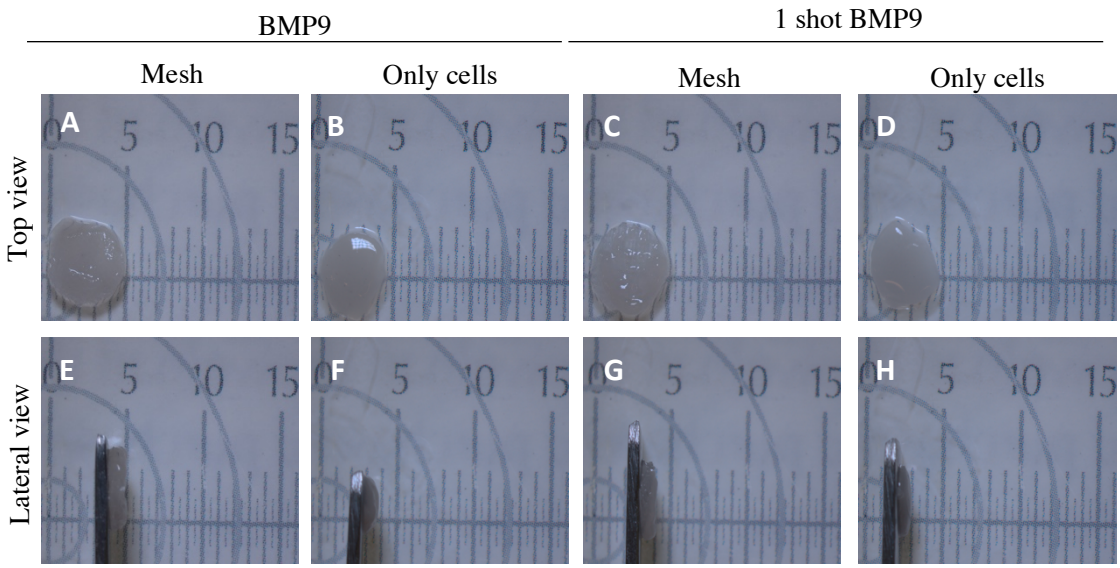


Supplementary figure 1. Macroscopic appearance of encapsulated ACPCs in NorHa+HAV hydrogels at day 7 and day 28 of culture. The chondrogenic differentiation medium contained BMP9 in A and D, TGFβ1 in B and E, and one shot of BMP9 the first day of culture in C and F.

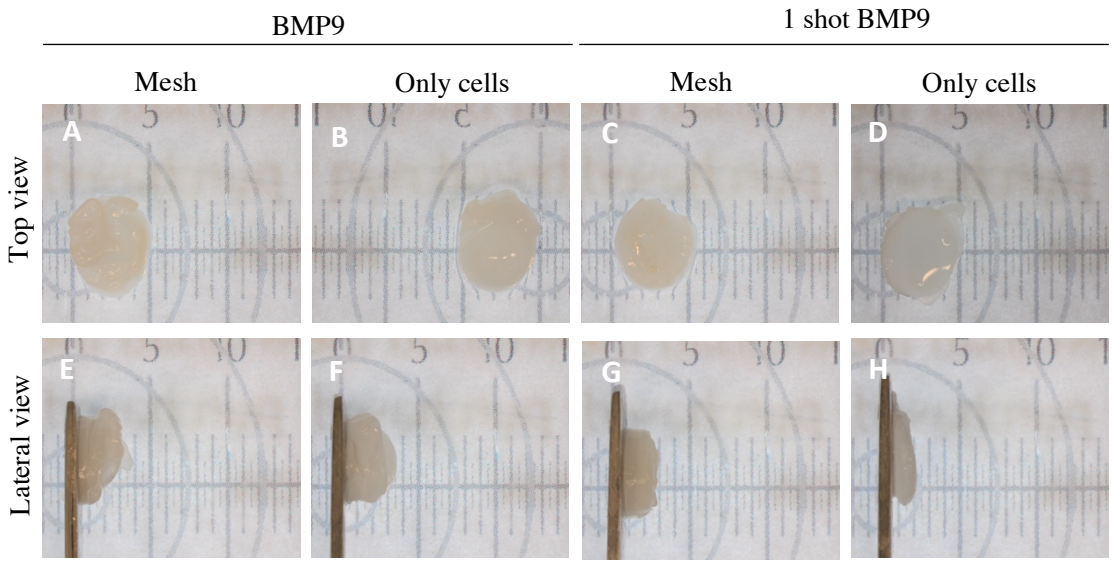


Supplementary figure 2. Macroscopic appearance of encapsulated ACPCs in NorHa hydrogels at day 7 and day 28 of culture. The chondrogenic differentiation medium contained BMP9 in A and D, TGFβ1 in B and E, and one shot of BMP9 the first day of culture in C and F.

ACPCs-laden PCL meshes



Supplementary figure 3. Macroscopic appearance of ACPCs-laden meshes (A and E, C and G) and ACPCs pellets (B and F, D and H) at day 7 of culture. The chondrogenic differentiation medium contained BMP9 (A, B, E, F) and one shot of BMP9 the first day of culture (C, D, G, H).



Supplementary figure 4. Macroscopic appearance of ACPCs-laden meshes (A and E, C and G) and ACPCs pellets (B and F, D and H) at day 28 of culture. The chondrogenic differentiation medium contained BMP9 (A, B, E, F) and one shot of BMP9 the first day of culture (C, D, G, H).