Universidad de Granada

TESIS DOCTORAL

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"NOVEL POLYMERS AND CHIMERIC LIGANDS WITH THERAPEUTIC PROPERTIES IN CHONDRAL PATHOLOGY"

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SUMMARY

Articular cartilage disorders including degenerative diseases such as osteoarthritis and rheumatoid arthritis are painful, debilitating and disabling clinical problems affecting millions of people worldwide. These lesions are currently sub-optimally treated in the clinics. Treatment of cartilage injuries remains a challenge due to the inherent biology of cartilage tissue, which limits their self-regenerating ability. New approaches in the field of tissue engineering (TE) are creating high expectations among the scientific community.

The objective of TE is the creation of a cellular environment that allows cells to function as they do in the native tissue, and for this purpose, the strategy generally involves: (i) an appropriate cell source, (ii) the maintenance of the cell phenotype that makes them functional, and (iii) the use of biocompatible materials that mimic the 3D structural architecture of the tissue. The concept of cell-based therapies for cartilage regeneration and repair is not new, since autologous chondrocytes implantation (ACI) and matrix-induced autologous chondrocytes implantation (MACI) are strategies that have been clinically used years ago. However, these techniques present important limitations: the appropriate cell numbers and differentiation state of the chondrocytes are difficult to maintain *in vitro* and, moreover, the integration of cells into surrounding tissue is not yet optimal.

In order to solve these problems, cartilage TE is focused on finding the appropriate cell source, the best growth factors and cytokines to induce chondrogenesis and maintain mature chondrocyte phenotype and, finally, the suitable 3D matrix with physicochemical and biologic characteristics that could allows a good integration and function into the native cartilage tissue.

The first objective of our study was to compare the chondrogenic potential of Nodal/BMP2 and Activin A/BMP2 chimeric ligands (NB260 and AB235) in adipose derived stem cells isolated from two different tissue sources, from lipoaspirates (ASCs) and from infrapatellar fat pad of patients with osteoarthritis (IFPSCs). *In vitro* experiments showed that cells in 3D pellet culture system exposed to NB260 and

AB235 acquired a characteristic chondrocyte phenotype and secreted extracellular matrix (ECM) similar to native cartilage that was confirmed by histological, immunological and molecular analysis. Moreover, *in vivo* experiments displayed that chimeric ligands-treated pellets integrated into the surrounding subcutaneous tissue in mice.

The second objective of this study was to induce redifferentiation of dedifferentiate chondrocytes isolated from osteoarthritis patients using the Activin A/BMP2 chimeric ligand (AB235) in a 3D pellet culture system. *In vitro* and *in vivo* experiments revealed that redifferentiated chondrocytes synthesized a cartilage-specific ECM primarily consisting of vertically-orientated collagen fibres and cartilage-specific proteoglycans. AB235-treated cell pellets also integrated into the surrounding subcutaneous tissue following transplantation in mice as demonstrated by their dramatic increase in size while non-treated control pellets disintegrated upon transplantation.

The third objective of this study was to evaluate a large number of different synthetic polymers to select a suitable polymer that favors the adhesion and proliferation of chondrocytes, and the maintenance in culture of a differentiated phenotype for prolonged periods of time. We proved that the hydrogel made by the crosslink of PA204 polymer seemed to mimic the 3D natural microenvironment of cartilage tissue. Chondrocytes growth into the PA204 hydrogel did not dedifferente, maintained a characteristic morphological and ultrastructural phenotype and produced an ECM composed by collagen II and proteoglycans typical of native cartilage tissue.

Summarizing, here we present a robust and extensive study in which we have demonstrated that Nodal/BMP2 and Activin/BMP2 chimeric ligands can induce chodrogenic differentiation in autologous adipose derived stem cells obtained from the infrapatellar fat pad of patients with osteoarthritis and from liposuctions.
Moreover, we have developed a suitable protocol to promote redifferentiation of autologous chondrocytes obtained from osteoarthritis patients in culture for long period of time and to induce the formation of a cartilage-like ECM that can integrate into the surrounding tissue in vivo. Finally, we have selected a new hydrogel based in

PA204 polymer to create a scaffold that proved to support chondrocyte growth and the production of a cartilage like ECM.

Since the success of cell therapies for cartilage injury depends on the quality and quantity of the implanted cells together with the 3D scaffold used to support the cells, the study presented here may have significant potential clinical applications.

RESUMEN

Los trastornos del cartílago articular, incluyendo enfermedades degenerativas como la osteoartritis y la artritis reumatoide, son dolorosos, debilitantes e incapacitantes, derivando en problemas clínicos que afectan a millones de personas en todo el mundo. Actualmente no hay un tratamiento óptimo desde el punto de vista clínico. El tratamiento de las lesiones del cartílago sigue siendo un desafío debido a la biología inherente del tejido cartilaginoso, la cual limita su capacidad de auto-regeneración. Por esto, los nuevos enfoques terapéuticos en el campo de la ingeniería tisular (TE) están generando grandes expectativas entre la comunidad científica.

El objetivo de la TE es crear un entorno celular que permita que las células funcionen como lo hacen en el tejido nativo, y para este propósito la estrategia a usar implica generalmente: (i) una fuente celular apropiada, (ii) mantenimiento del fenotipo celular que hace que sea funcional, y (iii) el uso de material biocompatible que imite la arquitectura estructural del tejido nativo. El concepto de terapia celular para la regeneración del cartílago no es nuevo, la implantación de condrocitos autólogos (ACI) y la implantación de condrocitos autólogos inducidos por la matriz (MACI) son estrategias que se han utilizado clínicamente desde hace décadas. Sin embargo, estas técnicas presentan limitaciones importantes: la obtención de un número adecuado de células, así como el mantenimiento *in vitro* del fenotipo de condrocitos maduros, además la integración del injerto en el tejido circundante todavía no es óptima.

Con el fin de resolver estos problemas, la TE dirigida al cartílago se centra en encontrar la fuente celular más apropiada, los mejores factores de crecimiento y citoquinas para inducir condrogénesis y evitar la des-diferenciación de los condrocitos maduros, y por último, encontrar la matriz 3D que reuna aquellas características fisicoquímicas y biológicas que permitan un buena integración y función de los condrocitos en el tejido del cartílago nativo.

El primer objetivo de nuestro estudio fue comparar el potencial condrogénico de los ligandos quiméricos Nodal/BMP2 y Activina A/BMP2 (NB260 y AB235) en células madre derivadas de tejido adiposo aisladas a partir de dos fuentes tisulares diferentes: de lipoaspirados (ASC) y de la grasa infrapatelar de la almohadilla de pacientes con

osteoartritis (IFPSCs). Los experimentos in vitro mostraron que las células cultivadas en sistemas de cultivo 3D en pellet expuestas a los factores NB260 y AB235 adquirieron un fenotipo característico de condrocitos y la matriz extracelular secretada (ECM) fue similar al cartílago nativo, lo que fue confirmado por estudios a nivel histológico, inmunológico y molecular. Además, en experimentos *in vivo* se demostró que los ligandos quiméricos con los que fueron tratados los sistemas de cultivo 3D en pellet se integraron en el tejido subcutáneo circundante en ratones.

El segundo objetivo de este estudio fue inducir la rediferenciación de los condrocitos desdiferenciados aislados de los pacientes con osteoartritis utilizando el ligando quimérico Activin A/BMP2 (AB235) en un sistema de cultivo 3D en pellet. Los experimentos *in vitro* e *in vivo* revelaron que los condrocitos rediferenciados sintetizaron una ECM cartilaginosa que consistia principalmente en fibras de colágeno orientadas verticalmente y proteoglicanos específicos del cartílago. Los pellets celulares tratados con los ligandos AB235 también se integraron en el tejido subcutáneo circundante después de su trasplante en ratones, como se demostró por su espectacular aumento de tamaño mientras que aquellos pellets control no tratados se desintegraron tras el trasplante.

El tercer objetivo de nuestro estudio fue la evaluación de un gran número de diferentes polímeros sintéticos con objeto de seleccionar un polímero adecuado para favorecer la adhesión y proliferación de los condrocitos, así como el mantenimiento en cultivo durante períodos prolongados de tiempo de su fenotipo diferenciado. Hemos demostrado que el hidrogel hecho por el entrecruzamiento del polímero PA204 parecía imitar el microambiente natural de la estructura 3D del tejido cartilaginoso. Los condrocitos crecidos en este hidrogel generado a partir del PA204 no producía desdiferenciación, manteniendo tanto las características morfológicas como el fenotipo ultraestructural, produciendo una ECM compuesta por colágeno tipo II y proteoglicanos típicos del tejido del cartílago nativo.

En resumen, esta tesis doctoral presenta un estudio sólido y amplio en el que hemos demostrado que los ligandos quiméricos Nodal/BMP2 y Activin A/BMP2 pueden

inducir la diferenciación condrogénica en células madre derivadas de tejido adiposo autólogo obtenido a partir de la de grasa infrapatelar de la almohadilla de pacientes con osteoartritis y de liposucciones. Por otra lado, hemos desarrollado un protocolo adecuado para promover la re-diferenciación en cultivos durante largos período de tiempo de los condrocitos autólogos obtenidos a partir de pacientes con osteoartritis y para inducir la formación de una ECM típica del cartílago que se pueda integrar en el tejido circundante *in vivo*. Por último, hemos seleccionado un nuevo hidrogel basado en el polímero PA204 que nos permite crear un "scaffold" que resultó óptimo para promover el crecimiento de los condrocitos y la producción ECM propia del cartílago.

Debido a que el éxito de las terapias celulares para la lesión del cartílago depende de la calidad y cantidad de las células implantadas junto con los scaffolds 3D utilizados, el estudio y los resultados aquí presentados pueden tener por tanto un importante potencial en aplicaciones clínicas.

INTRODUCTION

ARTICULAR HYALINE CARTILAGE

Articular cartilage is a highly specialized elastic connective tissue that covers joint surfaces of long bones. Its principal function is to provide a smooth, lubricated surface for articulation and to facilitate the transmission of loads with a low frictional coefficient, to ensure that joints and bones move together.

Unlike most tissues, articular cartilage is an avascular tissue, with no innervation or lymphatic vessels and it is primarily composed of a single cell type, the chondrocyte. Chondrocytes synthesize an abundant extracellular matrix (ECM), which suffers a strict regulation of matrix turnover in normal cartilage: a delicate balance between synthesis and degradation. The ECM of articular cartilage is characterized mainly by the presence of collagens (50-75%) and glycosaminoglycans (15-30%) which together with a high water content (78-80%) confer resistance to compression forces, providing at the same time flexibility and viscoelasticity properties (Salter 1998).

Articular cartilage has a limited capacity for repair due to: (i) the inability of the chondrocytes or articular progenitor cells to migrate towards the lesions, (ii) the limited proliferation capacity of chondrocytes in adults, and (iii) the absence of vasculature, which would allow progenitor cells from the blood or the bone marrow to migrate to the damage region and repair the tissue. These limitations have prompted researchers and clinicians to develop surgical methods to restore cartilage surfaces (Fosang & Beier 2011).

Microscopic Structure

Chondrocytes

Chondrocytes are the only cells found in cartilage, and constitute about 2% - 5% of total volume of articular cartilage. Chondrocytes are large cells, about 40 μm of

diameter, and differences in cell shape, number, size and metabolic activity have been found among the different regions of the cartilage tissue (Wong et al. 1996).

These highly specialized cells synthesize an extracellular matrix that isolates them in lacunaes preventing migration to other areas of cartilage and cell to cell contact. Due to this characteristic, chondrocytes receive nutrition through double diffusion barrier and live in a low oxygen concentration. Chondrocytes are anchored to the ECM by proteoglycans and membrane proteins such as syndecan, glypicans, anchorin CIi and integrins (Sophia Fox et al. 2009) (Bhosale & Richardson 2008). The balance between the synthesis and degradation of the ECM depends on the chondrocytes ability to detect changes in their surroundings. ECM turnover is controlled by cytokines and growth factors which direct the cellular machinery to synthesize matrix components, if ECM production is needed, or metalloproteinases (MMPs), hyaluronidases and aggrecanases, proteins involved in the degradation of the ECM(Demoor et al. 2014). Unfortunately, chondrocytes have limited potential for replication in adult articular cartilage, being this characteristic the main factor for the limited regeneration capacity of this tissue. Chondrocyte has a close relationship with its microenvironment, and its survival depends on an optimal chemical and mechanical environment (Sophia Fox et al. 2009)(Demoor et al. 2014).

Extracellular Matrix

Articular cartilage is composed by a dense ECM and the principal components are collagens, proteoglycans and water, although we can found other components, like non-collagenous proteins, lipids, phospholipids and glycoproteins, in lesser amount. All together, these components help to retain water within the ECM, which is critical to maintain its unique mechanical properties (Salter 1998).

Collagens

Collagens are the most abundant proteins in the ECM, and represents about 60% of the dry weight of cartilage tissue. Moreover, the collagen fibers constitute the endoskeleton of cartilage and provides the fibrillar network that endows cartilage with tensile strength.

Among the different types of collagens that form the ECM of cartilage tissue (collagen types I, II, IV, V, VI, IX, X, XI, XII, XIV and XVI), collagen type II (Col II) represents 90% to 95% of the total collagen. For this reason, Col II is being widely used as specific marker of chondrocytes together with aggrecan. Col II is a homotrimer composed of a α1 (II) chain that forms fibrils and fibers, like an endoskeleton, intertwined with proteoglycan aggregates and the others types of collagen, which help to stabilize the Col II fibril network (Martel-Pelletier et al. 2008).

The alteration in collagen structure or the amount of collagen present in the ECM can result in loss of functionality or cartilage injury. Deficiencies in collagen destabilize the ECM changing the visco-elasticity properties and resistance to compression forces of the healthy cartilage tissue. In fact, mutations in Col II have been identified as the cause of a number of rare chondrodysplasias being also implicated in some familial osteoarthritis types (Sophia Fox et al. 2009)(Demoor et al. 2014) .

Proteoglycans

Proteoglycans are heavily glycosolated protein monomers and constitute about 10% - 20% of the dry weight of cartilage and represent the second more abundant component of the ECM. The hydrophilic nature of the proteoglycan molecule attracts water absorption, which provides viscoelastic properties and flexibility to the articular cartilage facilitating the adaptation to compressive strength.

Proteoglycans have been classifying in: (i) large aggregating proteoglycan monomers (aggrecans), and (ii) small proteoglycans (decorin, biblycan and fibromodulin). The

molecule of proteoglycans is composed by disaccharide molecules called glycosamino glycans (GAGs) bound to a protein core through sugar bonds. Two main types of GAGs are distinguished chondroitin sulphate and keratan sulphate. When the proteoglycan is formed by more than 100 GAGs it constitutes an aggrecan molecule, the largest in size and the major structural component of cartilage along with Col II. Further, aggrecan can form large proteoglycan aggregates by crosslinking to hyaluronic acid (HA).

Aggrecan is found in the interfibrillar space of the ECM together with decorin and fibromodulin, which directly interact with Col II fibril. , Biglycan, meanwhile, is present in the surroundings of the chondrocytes (Knudson & Knudson 2001).

Water

Water is the most abundant component of articular cartilage, and it makes up about 80% of wet weight, providing nutrition and medium for lubrication and creating a low-friction gliding surface.

Water is not homogenous distributed in the cartilage tissue, in fact water content decreases from 80% in the superficial area to 65% in deeper areas. Most of the water is distributed in the extracellular space where different inorganic ions, such sodium, calcium and potassium chloride are dissolved (Martel-Pelletier et al. 2008)(Bhosale & Richardson 2008).

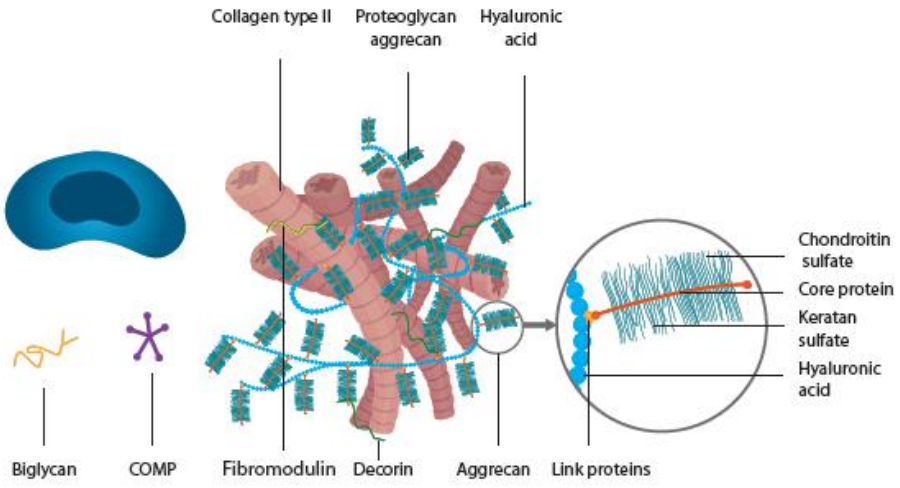

Fig. 1. Articular hyaline cartilage matrix: Microscopic structure.

Macroscopic Structure

Zones

The architecture of adult articular cartilage presents a typical distribution in four clearly differentiated zones: (i) superficial zone, (ii) transitional zone, (iii) deep zone, and (iv) calcified zone. Each of these zones present different characteristics based on amount of chondrocytes, ECM morphology and biochemical properties.

(i) Superficial zone, in contact with synovial fluid is composed basically by Col II and Col IX tightly packed and aligned parallel to the articular surface. The chondrocytes are abundant with flattened shape, locked in flattened lacunae. The superficial zone is the thinnest of all layers, representing about 10 - 20% of the total articular cartilage volume, and it protects the deeper layers from stresses and compression, thanks to its tensile properties.

(i) The transitional zone is formed by an abundant ECM, that is composed by proteoglycans and thicker collagen fibrils obliquely organized. Chondocytes are less represented than in the superficial zone, and have a spherical shape, locked in spherical lacunae. This middle zone represents about 40 – 60% of the total articular cartilage volume, and is the first line of resistance to compressive forces.

(ii) The deep zone contains the largest diameter collagen fibrils, which are disposed in a radial pattern, together with the highest proteglycan content and lowest water concentration. Chondrocytes have and spheroidal form and are arranged in columns that are perpendicular to the articulating surface. In this zone chondrocytes present a synthetic activity 10 times higher than chondrocytes embedded in the superficial zone. The deep zone represents approximately 30% of articular cartilage volume and is responsible to the resistance to compressive forces.

(iii) The calcified zone, in this region chondrocytes are presents in low number, express hypertrophic phenotype such as Col X, which turn the matrix into a calcified matrix.

The main role of this zone is to anchor the cartilage to bone, thanks to the collagen fibers of the deep zone that penetrate the calcified zone and bind with the periosteum.

Between the calcified zone and the deep zone, exist a thin border called ‘‘tidemark’’, which has a special affinity for basic dyes, such as toulidine blue. The tidemark serves as an important barrier between the uncalcified articular cartilage and the subchondral bone (Onyekwelu et al. 2009)(Temenoff & Mikos 2000)(Sophia Fox et al. 2009)(Bhosale & Richardson 2008).

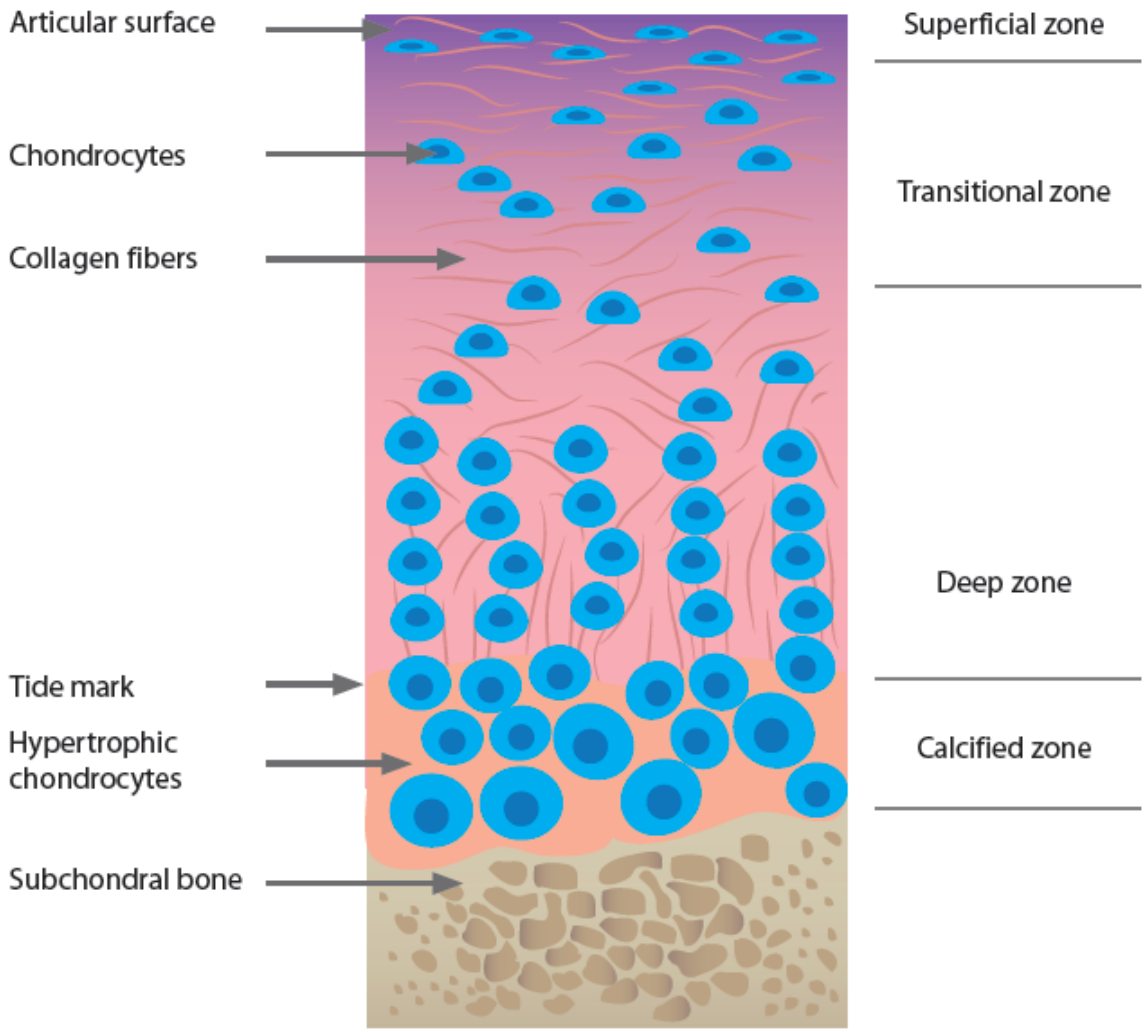

Fig. 2. Articular hyaline cartilage matrix. Macroscopic structure.

Extra cellular matrix regions

In each zone of the cartilage, it is possible to distinguish different regions in the ECM:

(i) pericellular region, (ii) territorial region, and (iii) interterritorial region. Differences between the three regions are based on proximity between chondrocytes, composition and collagen fibril diameter and organization.

(i) The pericellular region covers the area surrounding the chondrocytes that is in direct contact with the cell membrane. The main components are proteoglycans, glycoproteins, non-collagenous proteins, like cell membrane-associated molecule anchorin CII, and non-fibrillar collagens, including collagen type VI. This region provides the right environment for chondrocytes attachment to the ECM, and protects them from loads. Moreover, it serves as a transducer of biochemical and biomechanical signals to the chondrocyte (Wilusz et al. 2014).

(ii) The territorial region is found immediately after the pericellular region, surrounding it and the chondrocyte or a cluster of chondrocytes. It is thicker than pericellular region and it is composed by collagen fibrils, forming a spider web around the chondrocytes. The territorial region protects the chondrocytes against mechanical stresses.

(iii) The interterritorial region is the largest region with abundant proteoglycans and large collagen fibers. In this region collagen fibrils are differently orientated depending on the zone of the cartilage. Fibers are found arranged parallel to the surface on the superficial zone, disposed obliquely in the middle zone, and aligned perpendicular to the joint surface in the deep zone. This region is primarily responsible for the mechanical properties of the tissue (Onyekwelu et al. 2009)(Temenoff & Mikos 2000) (Bhosale & Richardson 2008).

Chondrogenesis

Cartilage development begins in the mesoderm by the condensation and differentiation of mesenchymal cells in pre-chondrocytes, as a result of specific cell-cell interactions that is a requisite step in chondrogenesis (DeLise et al. 2000). Then, pre-chondrocytes express specific cartilage transcription factors that induce the acquisition of a mature chondrocyte phenotype and the production of a characteristic cartilage ECM (Ikeda et al. 2004).

This process is mediated by the action of the transcription factors Sox9, L-Sox5 and Sox6 and the presence in the cellular microenvironment of several soluble factors such as some members of the transforming growth factor-β family (TFG- β), bone morphogenetic proteins superfamily (BMPs), insulin-like growth factor-1 (IGF-1) and fibroblast growth factor (FGF).

Sox9 is involved in the condensation phase of MSCs in pre-chondrocyte and in the beginning of the differentiation, stimulating expression of cartilage-specific markers (Col II, aggrecan and proteoglycans) and inhibiting cartilage vascularization, and endochondral ossification. Moreover, Sox9 induces the expression of others transcription factors such as L-Sox5 and Sox6, which definitively commit MSCs to develope into a chondrocyte lineage (Akiyama et al. 2002).

In the early stages of chondrogenesis, IGF-1 and BMPs stimulate growth by inducing proliferation of growth plate chondrocytes, but also by stimulating the differentiation of MSCs (Van Wyk & Smith 1999)(Reddi 2003). Also, members of the TFG- β family induce the synthesis of adhesion molecules such as syndecan, tenascin and fibronectin (Chimal-Monroy & Díaz de León 1999). On the other hand, the synthesis of Col II is stimulated by the action of TGF-β1, IGF-1 and BMP2 through the activation of Sox9 (Furumatsu et al. 2009)(Renard et al. 2012)(Uusitalo et al. 2001). FGF-2, which is a potent mitogen for adult articular chondrocytes (Trippel 2004), was proved to

stimulate chondrocyte mitogenesis and proteoglycan synthesis at low concentrations (Sah et al. 1994).

Likewise, hormones and other peptides have a role during chnondrogenesis. Parathyroid hormone-related peptide (PTHrP) for example, maintains chondrocytes in a proliferative state and inhibits their terminal differentiation into hypertrophic chondrocytes (Kronenberg 2006).

CHONDRAL PATHOLOGY: ORTEOARTHRITIS

Origin and development

The World Health Organization defines the osteoarthritis (OA) as: "a degenerative joint disease, which mainly affects the articular cartilage. It is associated with ageing and will most likely affect the joints that have been continually stressed throughout the years including the knees, hips, fingers, and lower spine region". It affects at 9.6% of men and 18.0% of women aged over 60, representing one of the ten most disabling diseases in developed countries.

The cause of OA is the alteration of the normal homeostasis of the cartilage due to mechanical and/or biologic events. The turnover between EMC synthesis and degradation is disrupted and the final balance leans towards ECM degradation. The first event is the alteration of the molecular structure and composition of the matrix, which is characterized by a loss of proteoglycans, an increase in water concentration, a decrease of aggrecan and Col II proteins, and the enhance expression of fibrotic makers such as collagens I, III, and V (Abramson et al. 2006).

OA is manifested by morphological, biochemical, molecular, and biomechanical changes in chondrocytes and ECM, which lead to softening, fibrillation, ulceration, loss of articular cartilage, sclerosis, and eburnation of subchondral bone, osteophytes, and subchondral cysts. As result of these alterations some clinical symptom are evident such as joint pain, tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of inflammation without systemic effects (Ge et al. 2006).

Factors involved in the development of osteoarthritis:

(i) Cytokines and growth factors such as Interleukins and the tumor necrosis factor (TNF) respectively, are key factors in OA development. These molecules are responsible for the production of proteases involved in cartilage destruction. In

addition, they act decreasing matrix metalloproteinases (MMPs) enzyme inhibitors, and inhibiting the synthesis of proteoglycans and Col II (Goldring 2000).

(ii) The activity of MMPs is also increased in OA due to an increment in the synthesis and activation of proenzymes by cytokines, others MMPs and/or plasmin, and the concomitant decrement of the activity of enzymes that inhibit MMPs (Naito et al. 1999). Likewise, it is possible to found a variety of other serine and cysteine proteinases (Huet et al. 1992).

(iii) A family of degradative enzymes called "disintegrin-like and metalloproteinase-like domains (ADAMs)" are highly regulated in OA, like ADAM-10 that has been found in the most fibrillated areas of OA cartilage (Chubinskaya et al. 2001).

(iv) Recently, two new ADAMs enzymes with an additional thrombospondin domain (ADAMTS) were discovered. ADAMTS-4 (aggrecanase 1) and ADAMTS-5 (aggrecanase 2) participate in aggrecan degradation (Song et al. 2007).

(v) Other molecular influences like fibronectin, can induce expression of MMPs and matrix degradation in chondrocytes. Through binding to fibronectin receptor is triggered the induction of enhanced gene expression of collagenase and stromelysin (Homandberg et al. 1992).

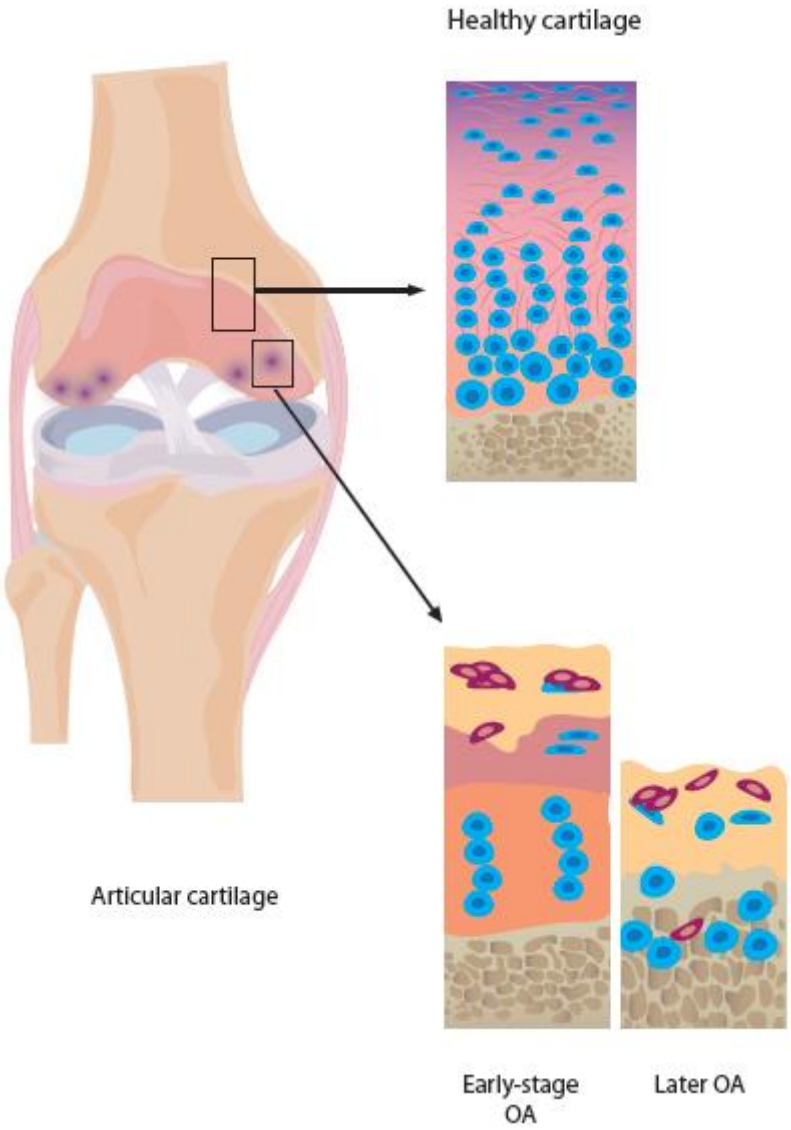

Fig. 3. Articular hyaline cartilage matrix: macroscopic structure in healthy and osteoarthritic (OA) zones.

Treatment

The progressive degeneration of OA is difficult to reduce and regenerate, and current interventions are aimed to relieve symptoms, reduce pain and control inflammation (Wieland et al. 2005).

Surgical treatments for cartilage repair in OA include osteochondral graft transplantation (mosaicplasty) and microfracture:

Mosaicplasty: was first described by Matsusue et al. (1993) and consists in a constructive technique that extracts cylindrical hyaline cartilage with the underlying subchondral bone of unaffected area for to be implanted on the chondral injury of 2-3 cm^2. Osteochondral cylinders are used to fill the chondral defect to create a 'mosaic' pattern hence called as mosaicplasty injury (Matsusue et al. 1993). The principal disadvantage of this technique is that the margins of the osteochondral plug are dead space, providing lower stability and may lead to degeneration of the tissue and failure of the graft (Evans et al. 2004).

Microfracture: it consists in the induction of small fractures in the subchondral bone by drilling small holes to allow blood and MSCs from bone marrow arrive into the lesion. This creates a blood clot containing MSCs that finally heal with scar tissue defect consisting of a mixture of fibrous tissue, fibrocartilage and hyaline cartilage (Gilbert 1998). This technique showed good results in young patients; however, the quality of the new tissue depends on many factors such as age, location or postoperative. Furthermore, the mechanical properties of scar tissue are lower compared to the native cartilage and joint may predispose an OA onset at medium and long term (Steadman et al. 2003).

These techniques relieve pain temporarily but are unsatisfactory in the long term and eventually fail (Hunziker 2002).

On the other hand, TE efforts such as autologous chondrocyte implantation (ACI) or matrix-induced autologous chondrocyte implantation (MACI) offer potential long-term solutions for regeneration of OA injuries.

Autologous chondrocyte implantation (ACI) was first described by Brittberg et al. (1994). The procedure involves the excision of a healthy biopsy by arthroscopy from a non load bearing region of the articular cartilage. The chondrocytes are then released

by enzymatic digestion and expanded in culture for later implantation in the injury (Brittberg et al. 1994).

Matrix-induced autologous chondrocyte implantation (MACI) is a variant of ACI and the difference consists in that, this technique instead of injecting chondrocytes as cell suspension, cells are cultivated in a bilayer collagen type I / type III porcine. Then, MACI membrane is directly secured to the lesion by fibrin glue without a cover (Nixon et al. 2015).

ACI and MACI displayed good results in long-term studies (Peterson et al. 2000) (Ronga et al. 2004), but presents important limitations: inability to treat large cartilage defects, thus excluding patients with OA. Better results have been obtained in young patients; however, the expensiveness of surgical procedures, donor site morbidity and the dedifferentiation of chondrocytes during *in vitro* expansion are the main disadvantages (Wu et al. 2013).

The habitual treatment in end-stage of knee OA is the replacement of articular surface by prosthesis; however, only elderly people (<60 years) are appropriate for this treatment.

TISSUE ENGINEERING FOR CHONDRAL PATHOLOGY

Robert Langer (2000) defines tissue engineering (TE) like "an interdisciplinary field that applies the principles of engineering and the life sciences to the development of biological substitutes that restore, maintain, or improve tissue function". The term regenerative medicine is often used synonymously with TE (Langer 2000).

The objective of TE is to create a cellular environment that allows the cells to function as they do in the native tissue, and for this purpose, the strategy generally involves: (i) an appropriate cell source, (ii) the maintenance of cell phenotype that makes them functional, and (iii) the use of biocompatible material. These three basic factors can be used individually or in combination, depending of the tissue to repair (Langer 2000) (Berthiaume et al. 2011).

Articular hyaline cartilage is an ideal candidate for TE because it has a limited capacity for repair due to the absence of vasculature, which would allow progenitor cells from the blood or the bone marrow to enter the tissue. Moreover, the resident articular progenitor cells or chondrocytes entrapped within the surrounding matrix do not migrate into the lesions to secrete a reparative matrix. The concept of cell-based therapies for cartilage regeneration and repair is not new, ACI and MACI techniques has been used clinically since decades, but presents important limitations as mentioned above. Furthermore, cartilage obtained with these techniques often lacks the structure of native cartilage and is composed by Col I instead of Col II (fibrocartilage), which is biochemically and biomechanically inferior to hyaline cartilage (Chung & Burdick 2008).

The TE in the field of cartilage regeneration is working in:

(i) The search of the best cell candidates between chondrocytes or MSCs, in terms of readily available sources for isolation, expansion and repair potential.

(ii) Identifying growth factors and/or the appropriate manner of application that promote chondrogenic differentiation and chondrocyte phenotype maintenance.

(iii) The search of biocompatible and biodegradable natural or synthetic matrices as cell carriers and chondrogenic factors releasing that present a good adaptation to the lesion area and mechanical demand of this tissue (Vinatier, Bouffi, et al. 2009).

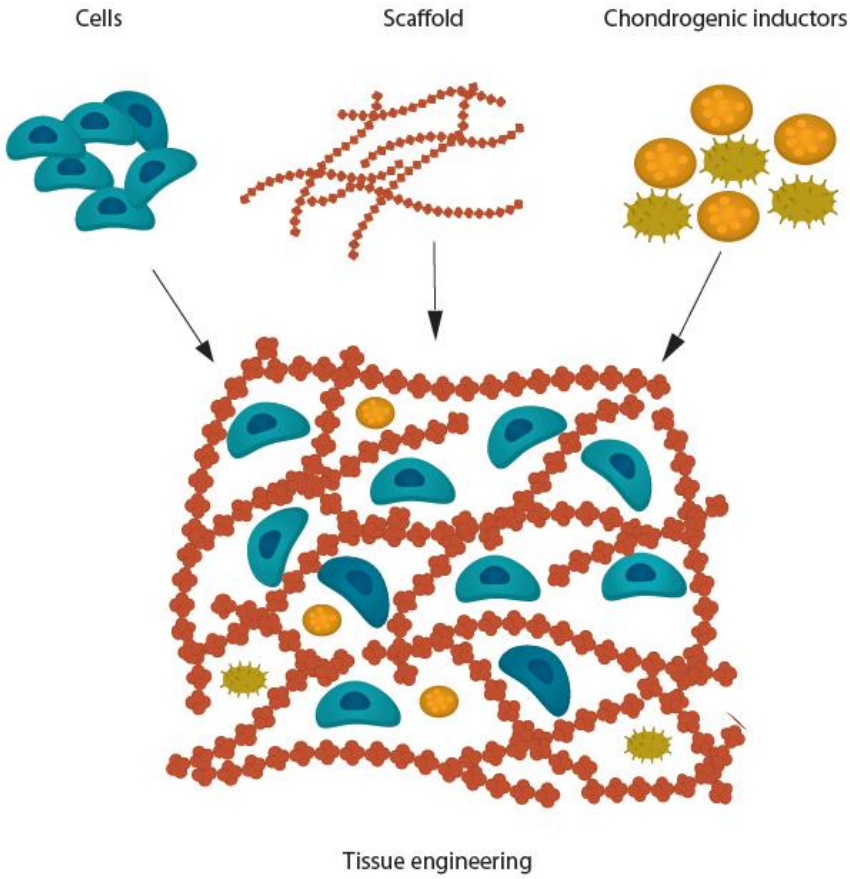

Fig. 4. Requirement for cartilage tissue engineering: combination of cell, chondrogenic inductors and 3D support.

Cell source for cartilage tissue engineering

Chondrocytes, fibroblasts, stem cells and genetically modified cells have all been explored for their potential as a viable cell source for cartilage repair. In order to choose the best source, the neo-cartilage generated must be of good quality and have not only a biochemical composition and a zonal organization of the neo-synthesized molecules, but also a function (such as mechanical behaviour) close to that of the native tissue.

Chondrocytes

Chondrocytes appear to be the logical cell of choice to repair cartilage injures, because of are the unique cell type in mature articular cartilage, and are responsible for the secretion and maintenance of ECM. However, the use of chondrocytes is limited by two major concerns.

Chondrocytes constitute only about 2 - 5% of the total volume of articular cartilage, whereby the number of chondrocytes that can be isolated from a patient biopsy is very restricted. In order to increase the number of cells, freshly isolated chondrocytes are cultured and expanded *in vitro*; however, this leads to the problem of chondrocyte phenotype loss due to cell dedifferentiation that occurs during prolonged monolayer culture. This dedifferentiation causes that chondrocytes lose their round shape and become flattened fibroblast-like cells with an increased proliferative capacity that is accompanied by changes in gene expression and surface markers including decreased Col II and Aggrecan, and increased levels of Col I and Col X (Chung & Burdick 2008) (Ma et al. 2013). Between other, these limitations are the responsible that ACI implants often lacks the structure of native cartilage and create fibrocartilage instead of hyaline cartilage.

A variety of methods have been used to prevent or revert dedifferentiation state such as three dimensional (3D) culture and scaffolds, bioreactors, exogenous mechanical

and biomechanical stimulation, reduced oxygen tension and addition of growth factors (Homicz et al. 2003)(Das et al. 2015)(Kurz et al. 2004) (Mandl et al. 2002)(Claus et al. 2010).

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) have been considered as an alternative source of cell in cartilage TE. MSCs can be easily isolated from different sources, such as bone marrow, umbilical cord blood, cord blood, and adipose tissue. MSCs presents high *in vitro* capacity of expansion, and the principal characteristics are the ability to adhere to plastic, specific phenotype (CD73, CD90 and CD105), and potential to differentiate into mesoderm linage (adipocytes, chondrocytes and osteblasts) (Dominici et al. 2006).

Numerous methodologies based in growth factors and/or 3D culture systems promote that MSCs undergo chondrogenesis. In general, the most common growth factors for chondrogenic induction of MSCs come from the TGF-β superfamily (Puetzer et al. 2010), that usually are in combination with various biomaterials from different origin (Awad et al. 2004). Even it had been demonstrated that substrates can induce chondrogenesis in MSCs by itself, in the absence of any chondrogenic inductor (Glennon-Alty et al. 2013). Likewise, co-culture, conditioned medium and chondrocytel extract methods can induce chondrocyte differentiation of MSCs (Levorson et al. 2014) (Alves da Silva et al. 2015) (López-Ruiz et al. 2013).

Pellets (Markway et al. 2010) or micromass (Giovannini et al. 2010) culture systems stimulate chondrogenic differentiation of MSCs, because promote cell–cell contact and help to form aggregates, and MSCs packed in high cell density can mimic the mesenchymal condensation during embryologic chondrogenesis (Lefebvre & Smits 2005).

Limitations of MSCs are the amount of matrix produced and mechanical properties, which can be inferior to that produced by chondrocytes (Mauck et al. 2006). However, the intra-articular injection of MSCs proved to be effective in terms of reducing pain,

improving tissue function and a robust capability to regenerate hyaline-like cartilage, just as no report of tumor formation at the implant sites (Wakitani et al. 2011) (Pak et al. 2013).

Embryonic stem cells

Embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts present two important advantages: a high rate of proliferation and the ability to differentiate into all somatic cell types.

For chondrogenesis, ESCs must pass through an aggregation stage of embryonic bodies (EBs) before differentiation, although, recently studies had been demonstrated that it is possible to induce chondrogenic differentiation without EBs formation (Nakagawa et al. 2009) (Hwang et al. 2008). The use of TGF-β superfamily ligands in combination with 3D system cultures induced chondrogenesis in ESCs, with ECM deposition characteristic of native cartilage (Hwang, Varghese, et al. 2006)(Hwang, Kim, et al. 2006). Moreover, the microenvironment created by chondrocytes in co-culture with ESCs was shown to induce chondrogenesis (Vats et al. 2006).

The use of ESCs for clinical applications is complicated due to the capacity of these cells to develop *in vivo* tumors, as well as antigenicity and ethical issues (Hipp & Atala 2008).

Fibroblasts

Fibroblasts have capacity to differentiate into chondrocytes when cultured under the appropriate conditions. The skin, the higher organ, present a minimally invasive and abundant fibroblast source, so fibroblast can constitute an alternative cell type in cartilage TE.

Just as other cell sources, fibroblast can differentiate towards chondrocyte phenotype under the action of specific growth factors and environment. Recently, Moradi et al.

(2001) demonstrated the potential of bovine articular derived cartilage matrix combined with TGF-β3 in porous scaffolds to induce chondrogenesis in human fibroblasts (Moradi et al. 2015). Similar results were obtained by Nicoll et al. (2001) that achieve the expression of cartilage matrix proteins when cultured fibroblast in high density micromass with lactic acid (Nicoll et al. 2001).

Induced pluripotent stem cells (iPSCs)

Yamanaka et al. (2006) demonstrated that somatic cells can be reprogrammed into pluripotent cells by using transcription factors associated with pluripotency (Takahashi & Yamanaka 2006). Induced pluripotent cells, called iPSCs cells, have similar characteristics to ESCs while its use does not raise ethical and political dilemmas.

The potential use of iPSCs to restore damaged cartilage was showed by iPSCs from OA chondrocytes and adult fibroblast with the capacity to increase cartilage markers, Col II, aggrecan and COMP, and to avoid the hypertrophic phenotype with low levels of Col I and Col X, after co-culture with chondrocytes and exposition to BMP4, respectively (Wei et al. 2006) (Wei et al. 2012)(Diekman et al. 2012). The expression of chondrogenic markers in human iPSCs was shown to be either comparable or superior to bone marrow MSCs, and repair damaged cartilage with high quality in rat ostechondral defect model (Ko et al. 2014).

However, cell therapy with these cells is still far from being put into clinical practice, since it is necessary to study in more depth and resolves the problem of possible teratomas formation.

Genetically modified cells

Genetically modified cells that induce or maintain condrocytic phenotype are being widely studied. For this purpose, over /down-regulation of numerous mechanisms of action and genes are candidates: inhibition of catabolic pathways, stimulation of anabolic pathways and/or cytoprotection/proliferation. As well as, different cell

sources were studied: chondrocytes, MSCs, fibroblast, synovial cells... (Madry & Cucchiarini 2011).

Shuer et al. (2000) avoid the deffirentiation process and enhanced the expression of proteoglycans and Col II with the incorporation of TGF-β1 in chondrocytes (Shuler et al. 2000), and similar results were observed when chondrocytes from MSCs could be obtained by the use of vectors with BMP-2 (Carlberg et al. 2001). Transplantation of genetically modified cells into *in vivo* models has been successful (Madry & Cucchiarini 2011). For example, modified fibroblasts expressing active TGF-β1 were injected into cartilage defects in rabbits and showed evidence of newly formed hyaline cartilage after 6 weeks (Lee et al. 2001) and, recently, Ivkovic et al. (2010) modified autologous bone marrow cells using adenovirally-transduced cells with a TGF-β1-expressing vector to treat partial thickness chondral defects in sheep, improving histological, biochemical and biomechanical parameters (Ivkovic et al. 2010).

Even, in the early 1990s, initiation of clinical trials based on the transfer of an interleukin-1 receptor antagonist (IL-1Ra) gene sequence by retroviral vector into synovial cells for the treatment of arthritis, displayed good results with active transgene product expressed and biologically active. This study confirmed the safety, feasibility, and efficacy of genetically modified cells to treat chondral injuries (Evans et al. 1996)(Evans et al. 2005).

Chondrogenic inductors

During the development and homeostasis of cartilage, several growth factors, cytokines and transcription factors are involved. In TE, chondrogenic inductors have been employed to induce, accelerate, maintain, and/or enhance cartilage formation. They exist different ways to apply stimulating factors, for example, like additives to culture media, incorporated into scaffolds for programmed delivery or by transfection.

The most characterized and employed factors that stimulate chondrogenesis and ECM synthesis include TGF-β, BMPs, FGFs and IGF-1.

Transforming Growth Factor-β family (TGF-β)

Members of TGF-β family have been shown to play a major role in cartilage development, and probably belong to the most investigated biologically active substances within the field of cartilage TE. TGF-β superfamily consists of 33 members that bind with TGF-β receptor, and can be generally divided into TGF-β, BMP, growth and differentiation factor (GDF), activin and inhibin, nodal, Mullerian inhibiting substance (MIS), and glial cell line-derived neurotrophic factor (GDNF) subfamilies. TGF- β superfamily ligands can be divided into two subfamilies by the two separate Smad pathways: TGF-β subfamily that include TGF-β, Nodals, Activins, myostatin (GDF-8) and Mullerian inhibiting substance, and activate Smad2/3 transcription factors. On the other hand, bone morphogenetic protein (BMP) subfamily ligands that consist in BMPs and GDFs that activate Smad1/5/8 transcription factors (Kwiatkowski et al. 2014).

The TGF-β subfamily ligands includes 5 members (TGF-β1–5) where TGF-β1, 2 and 3 are generally considered to be potent stimulators of proteoglycans and Col II synthesis in primary chondrocytes and it has been shown to enhance *in vitro* proliferation and redifferentiation of chondrocytes (Das et al. 2015)(Lee et al.)(Klangjorhor et al. 2014) and, also, *in vivo* chondrogenic induction of MSCs (Fan et al. 2007)(X. Wang et al. 2014). Interestingly, Activin A and Nodal enhanced chondrogenesis of MSCs (Jiang et al. 1993)(Peran et al. 2013)(Esquivies et al. 2014).

BMPs subfamily regulates chondrogenesis and ECM composition (Chen et al. 2004). BMP2, BMP4 and BMP6 enhance the synthesis of the two major constituent of cartilage ECM, Col II and aggrecan (Gründer et al. 2004)(Miljkovic et al. 2008)(Estes et al. 2006). Moreover, BMP2 upregulated the expression of Sox9, a key marker for early chondrogenesis (Yoon et al. 2005). BMP7 stimulates the production of proteoglycan rich ECM. BMP7, BMP2 and BMP4 can direct differentiation towards chondrocyte phenotype of MSCs and enhance *in vivo* chondrogenesis with significantly improved articular cartilage repair (Di Cesare et al. 2006)(Kuroda et al. 2006)(Kuo et al. 2006).

Fibroblast Growth Factors family (FGF)

The fibroblast growth factor (FGF) family comprises 22 structurally related proteins that bind one of four FGF receptors (FGFRs). Abnormalities in the skeletal development as a consequence of mutations in the genes encoding FGFRs, highlighted the importance of the FGF signaling (Vinatier, Bouffi, et al. 2009).

FGF-2 is a mitogen that has been used to preserve the chondrogenic potential of monolayer expanded chondrocytes and to increase cell proliferation, which in turn can result in greater ECM deposition and accelerated repair (Chung & Burdick 2008). In addition, FGF-2 and FGF-18 induce chondrogenesis of MSCs in a 3D culture system (Solchaga et al. 2005)(Davidson et al. 2005), and can stimulate *in vivo* articular cartilage restoration (Takafuji et al. 2007)(Moore et al. 2005).

Insulin-like Growth Factors family (IGF)

The insulin-like growth factors (IGF) family is composed of two ligands (IGF-1 and IGF-2) and two cell surface receptors (IGF1R and IGF2R). IGF-1 is considered the main anabolic growth factor and mediator of cartilage homeostasis that promotes proteoglycan and Col II synthesis and chondrocyte survival and proliferation. It was demonstrated that IGF-1 also induces chondrocytic phenotype in MSCs as shown by the upregulation of the specific markers. Better results were obtained when IGF-1 treatment of MSCs was combined with other growth factors, mainly with TGF- β1, BMP2 and BMP7, because they have an additive effect, which leads in a significant increase of cartilage matrix synthesis (Danišovič et al. 2012). *In vivo*, in cartilage defect horse model, defects filled with fibrin clots loaded with IGF-1 repaired better than empty defects and contained mainly chondrocytes with predominantly Col II rich matrix (Schmidt et al. 2006).

Scaffolds for cartilage tissue engineering

Chondrocytes naturally reside embedded in a 3D network, and this 3D environment allows dynamic interaction between cells and the ECM that facilitates signals for growth, differentiation, survival and organization of the cells present in the tissue (Hynes 1999). The main goal of cartilage TE is the development of cell carriers reproducing 3D structures with size, architecture and specific physical properties that mimic the natural environment of native cartilage in order to promote certain functions such as cell adhesion, mobility and cell differentiation (Engel et al. 2008).

Scaffolds must gather a number of important features. They should: i) have controlled degradation and have non toxic degradation products; ii) be highly porous with an interconnected pore structure that allows the diffusion of nutrients and waste products; iii) have suitable physical and mechanical properties and, iv) promote cell adhesion, viability, proliferation and differentiation, as well as ECM production (Chung & Burdick 2008). Additionally, handling facilities, the manufacturing process and costs should be taken into account.

Moreover, scaffolds can incorporate additional functions such as specific nanotopographies, protein coatings or chemical conjugation of specific signaling molecules (Chung & King). For example, polyacrylate substrates can induce chondrogenesis in MSCs by itself, in the absence of any chondrogenic inductor by the action and distribution of integrin-binding Arg-Gly-Asp (RGD) motif in the surface (Glennon-Alty et al. 2013), or the MSCs chondrogenic differentiation by the controlled release of TGF-β factors encapsulated, immobilized or merged in the scaffolds (Kim et al. 2015)(Zheng et al. 2015).

Polymers shaping the scaffolds can be divided into natural and synthetic based on their origin.

Natural polymers

Natural scaffolds can be directly extracted from plants, animals, or human tissues or derived thereof, and are based on components of the ECM. Among the most employees in cartilage engineering are collagen, alginate, agarose, chitosan, hyaluronic acid, chondroitin sulfate and silk fibroin.

Collagen and hyaluronan-based matrices are the most popular natural scaffolds with clinical application because they are normal constituents of articular cartilage. Collagen is a fibrous protein and hyaluronic acid is a GAGs which links with collagen to form a network of ECM that provides mechanical support to the connective tissues and to be a template for cell distribution. *In vitro*, it had been demonstrated the potential of collagen and hyaluron scaffolds to induce chondrogenesis or to maintain chondrocyte phenotype in long culture periods (Freyria et al. 2009)(Mukaida et al. 2005)(Grigolo et al. 2002). Furthermore, membranes formed of Col I/III and hyaluronic acid are clinically available for ACI (Vinatier, Mrugala, et al. 2009).

Scaffolds based on polysaccharides of different source, such as agarose (algae), chitosan (exoskeleton of crustaceans and insects) or alginate (algae), have been demonstrated the *in vitro* potentital to induce chondrogenesis in MSCs (Shen et al. 2009) and dedifferentiated chondrocytes (Domm et al. 2004) (Lee & Shin 2007), as well as, to repair *in vivo* articular cartilage defects in rabbit knees, with appropriate integration in the surrounding tissue and high production of Col II and GAGs (Rahfoth et al. 1998).

Silk fibroin is a fibrous protein that is obtained mainly from cocoons of *Bombyx mori* silkworms. It is a natural polymer that combines the most desirable physic-chemical and biology properties to produce scaffolds, and can be processed into as fibers, hydrogels, films, foams... (Pina et al. 2015) (Yang et al. 2012). Numerous studies support the role of silk scaffolds in TE oriented to repair cartilage injuries (Meinel et al. 2004)(Kawakami et al. 2011)(Yan et al. 2015).

Some studies also combine different natural biomaterials to increase the chondrogenic potential of biomaterials. Some examples include the combination of chitosan with hyaluronic acid (Correia et al. 2011), collagen with hyaluronan (Meng et al. 2014), and silk fibroin with chitosan (Silva et al. 2008).

Natural polymers have some limitations such as may stimulate the immune system response, the degradation very quickly, and less adequate mechanical properties (Lee & Shin 2007).

Synthethic polymers

Synthetic polymers are interesting in TE due to their high versatility, properties, reproducibility and good workability (Gunatillake & Adhikari 2003). This material is more suitable to adapt their characteristics for cartilage regeneration, since their degradation can be better controlled and are easier to mold with the desired porosity, or can be processed more easily with more predictable results (Capito & Spector 2003). Furthermore, they presents the ability to add functional chemical groups to enhance the chondrogenic potential of the material (Guo et al. 2008).

Poly (ethylene glycol) (PEG) and Poly (α- hydroxypoly ester), which includes Poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and the copolymer poly (lactic-co-glycolic acid) (PLGA), are the most commonly used synthetic biodegradable polymers for cartilage TE. *In vitro*, chondrocytes plated onto these polymers secreted dense cartilage matrix to fill the void spaces in the scaffold (Park et al. 2004) (Zwingmann et al. 2007) (López-Ruiz et al. 2013); whereas *in vivo*, they produced an ECM composed by Col II and GAGs and the structure was closely resembled cartilage histologically (Freed et al. 1993) (Zwingmann et al. 2007). In clinic, BioSeed-C is a porous 3D scaffold made of PGA and PLA that has been reported to induce the formation of hyaline cartilage, which is associated with a significant clinical improvement of joint function (Kreuz et al. 2011).

Poly(e-caprolactone) (PCL) is a degradable semicrystalline aliphatic polyester that is characterized by its biocompatibility, good mechanical properties, slow degradation

rate and easy fabrication (Lam et al. 2009). Numerous studies have demonstrated the biocompatibility, differentiation, and specific gene expression of chondrocytes seeded into PCL scaffolds subcutaneously implanted or in cartilage defects (Jung, Kim, et al. 2008) (Jung, Park, et al. 2008). PCL scaffolds have been widely used in cartilage TE because maintained their mechanical integrity after implantation and guided *in vivo* cartilaginous tissue growth. Moreover, degradation product of PCL is known to be biocompatible, and the mechanical properties of the regenerated tissue matched those of normal articular cartilage (Martinez-Diaz et al. 2010).

Polyurethanes (PU) cover a wide family of elastic polymers containing urethane bonds in the polymer chain (Carletti et al. 2011). PU have excellent physical properties and good biocompatibility making them good candidates for many different biomedical applications and, in cartilage restoration, specifically can promote the formation of a functional cartilage-like ECM (Grad et al. 2003) (Tsai et al. 2015).

On the other hand, polyacrylates (PA), a family of polymers that consist in ester acrylate monomers containing vinyl groups, are less extended in the area of cartilage restoration, but a recent publication demonstrated the potential of PA substrates modelled on the functional group composition and distribution of the Arg-Gly-Asp (RGD) integrin-binding site to induce chondrogenesis in MSCs in the absence of other exogenous stimulus (Glennon-Alty et al. 2013).

Despite the advantages mentioned above, synthetic material presents some important limitations. It is very important take into account polymer surface characteristics and composition, which are necessaries for direct cell-scaffold interactions and play an essential role in adhesion, cell signaling, directed degradation and matrix remodeling (Allen et al. 2006). In addition, products resulting from the degradation process may be toxic or elicit an inflammatory response (Middleton & Tipton 2000).

The combination of natural and synthetic polymers allows to exploit the advantages of each, since the composition, architecture (pore size, porosity and shape), and mechanical properties of the biomaterials affect chondrogenesis. In this sense, we can

found numerous examples, being the use PCL and chitosan the most common, combined between them or with another material (Neves et al. 2011) (Chen et al. 2014) (Yang et al. 2012).

In order to regenerate osteochondral defects, several authors have echoed the need of create biphasic media that combine a phase for chondral and osteogenic injuries (Liu et al. 2013) (Shimomura et al. 2014). Recently, it has been published a novel bilayer scaffold that combine a silk layer and silk-calcium phosphate layer (silk/silk-nanoCaP), that favors the cartilage regeneration (silk layer), osteogenesis and angiogenesis (silk-nanoCaP layer) when rabbit bone marrow mesenchymal stromal cells were seeded on it and implanted in an *in vivo* model of osteochondral injury (Yan et al. 2015). In clinic, various scaffolds are being tested such as scaffolds that combine collagen and hydroxyapatite (Kon et al. 2014) or PLGA (Chiang et al. 2013). However, these studies showed controversial results, whereas some authors have reported a favorable short-term clinical outcome and an improvement of the imaging appearance of the osteochondral implant over time, others have reported poor results (Kon et al. 2014) (Bedi et al. 2010) (Carmont et al. 2009).

HYPOTHESIS

The articular cartilage is especially vulnerable to damage and has poor potential for regeneration because of its intrinsic characteristics. Progressive degeneration of articular cartilage leads to joint pain and dysfunction, as part of the clinical syndrome of OA. The OA is one of the ten most disabling diseases in developed countries, with high socio-economic impact. Therefore, the need to develop novel therapeutics strategies has increased in the last years.

The regeneration of damaged articular cartilage is based in three pillars: (i) cell-based therapy, (ii) the use of a 3D support for cells implantation and, finally, (iii) the use of growth factors. These three strategies join forces to maintain chodrocytic phenotype, proliferation and the production of mature ECM.

The present work is based on the following hypothesis:

1. Adipose derived stem cells are multipotent somatic highly proliferative cells with inherent chondrogenic differentiation potential in the presence of appropriate growth stimuli, along specific pathways for producing cartilage tissue. Moreover, 3D culture systems in combination with chondrogenic inductors such as TGF-β superfamily ligands, constitute a suitable 3D environment that enhances the differentiation towards mature chondrocyte phenotype. Here, we hypothesize that adipose derived stem cells isolated from the infrapatellar fat pad (IFPSCs) of OA patients and from liposuctions (ASCs) of patients undergoing a liposuction surgery can undergo chondrogenesis when stimulated with Nodal/BMP2 (NB260) and/or Activin/BMP2 (AB235) chimeric ligands in 3D pellet culture system, with the production of appropriate cartilage ECM.

2. Autologous chondrocytes should be the most appropriate source for TE of chondral pathologies, but it is difficult to obtain they in big amounts and present the problem of dedifferentiation when are expanded in monolayer culture during long periods. Chondrocytes have the capacity to revert the dedifferentiation state to mature phenotype under proper conditions, such 3D culture systems and/or chondrogenic

inductors. We hypothesize that Activin/BMP2 (AB235) chimeric ligand in combination with a 3D pellet culture system induce redifferentiation of functional OA patient-derived dediferentiated chondrocytes.

3. Chondrocytes cultured in 3D systems can retained their mature phenotype in prolonged culture preventing the dedifferentiation process, producing characteristic ECM and providing a 3D environment suitable for TE. Moreover, polymer microarrays are a high-throughput system that allows screening hundreds to thousands different polymers. We hypothesize that polyurethanes and polyacrylates gather the proper fisico-chemical, mechanics and biological characteristics for adhesion, proliferation and maintaining mature phenotype of functional OA patient-derived chondrocytes during long periods in culture.

OBJECTIVES

The main objectives of this study are:

1. To induce chondrogenic differentiation of ASCs obtained from liposuction surgery and IFPSCs obtained from patients with OA using Nodal/ BMP2 (NB260) and Activin/BMP2 (AB235) chimeric ligands in 3D pellet culture system.

2. To induce redifferentiation of dedifferentiated chondrocytes obtained from patients with OA using Nodal/BMP2 (NB260) and Activin/BMP2 (AB235) chimeric ligands in 3D pellet culture system.

3. To screen polyurethanes and polyacrylates based polymers using the high-throughput system polymers microarrays for the adhesion, proliferation and long periods in culture of chondrocytes obtained from patients with OA.

MATERIAL AND METHODS

1. Tissue samples

1.1. Articular cartilage tissue

Articular cartilage was obtained from patients with knee OA during joint replacement surgery, isolated from the femoral side, selecting the non-overload compartment: lateral condyle in varus knees and medial condyle in the valgus cases (leaf shape; size: length: 16 ± 5 mm and width: 8 ± 2 mm). None of the patients had a history of inflammatory arthritis or crystal-induced arthritis and only cartilage that macroscopically looked relatively normal was used for this study. Samples collected at joint arthroplasty were transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 100 U/ml penicillin and 100 mg/ml streptomycin. Informed patient consent was obtained for all samples used in this study and samples were collected in accordance with the Research Ethics Committees of the University Hospital of Malaga, "Virgen de la Victoria" and University of Granada (ES180870000164).

1.2. Adipose tissue from Hoffa's fat pad

Hoffa's fat pad was harvested from the interior of the capsule excluding vascular areas and synovial regions. Samples were collected at the moment of joint arthroplasty from OA patient, after signed an informed consent, approved by the Ethics Committee of the Clinical University Hospital of Málaga, Spain. Samples were maintained in DMEM with 100 U/ml penicillin and 100 µg/ml streptomycin until processing.

1.3. Subcutaneous adipose tissue

Subcutaneous adipose tissue was obtained from patients undergoing liposuction procedure after informed consent from all patients and approval from the Ethics Committee of Clinical University Hospital of Málaga, Spain.

2. Isolation and culture of primary cells

2.1. Isolation and culture of human articular chondrocytes

Articular cartilage was minced and digested overnight in 0.08% collagenase IV (Sigma) digestion at 37°C with gentle agitation. Cells were centrifuged and rinsing to remove the collagenase. The remaining cells were then plated in cultured flasks with chondrocytes media: DMEM (Sigma) supplemented with 20% fetal bovine serum (FBS, Gibco), 5 ml of 1% ITS (Insulin-Transferrin-Selenium, Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. After 24 hours the medium was replaced with fresh medium supplemented with 10% FBS. At 80% of confluency cells were detached with TrypLE (Invitrogen) and subcultured.

2.2. Isolation and culture of adipose derived stem cells

Samples of adipose tissue from lipoaspirates or from Hoffa's fat pad tissue were finely minced and digested using an enzymatic solution of 1 mg/ml collagenase type IA (Sigma) at 37 ºC for 1 h on a shaker. After digestion, collagenase was removed by a single wash in sterile PBS, followed by two further washes in DMEM supplemented with 10% FBS. The cell pellet was resuspended in DMEM (Sigma) containing 10% FBS and 1% penicillin/ streptomycin and cultured at 37ºC in 5% CO_2. After 48 hours the medium was removed to discard non adherent cells. At 80% of confluency the cells were released with TrypLE (Invitrogen) and subcultured.

3. Cell characterization

3.1. Flow cytometry analysis of IFPSCs and ASCs

The immunophenotype of IFPSCs and ASCs was analyzed by flow cytometry (FACS). Cells were washed and resuspended in phosphate buffered saline (PBS) with 2% bovine serum albumin BSA (Sigma, St. Louis, MO), and 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma). Cells were incubated in dark for 30

minutes at 4º C with the appropriate fluorochrome-conjugated monoclonal antibodies. The markers used were: CD73-APC, CD90-FITC, CD105-PE, CD34-PE, CD45-PerCP, and CD133-APC (Miltenyi). All cells were washed in PBS and analyzed in a FACS Canto II cytometer (BD Biosciences).

3.2. Differentiation assays of adipose derived stem cells

ASCs isolated from lipoaspirates or for Hoffa's fat pad tissue were plated at 2 x 10^3 cells/cm^2 in DMEM (Sigma) containing 10% FBS (Gibco) with penicillin and streptomycin at 100 µg/ml (Sigma) and allowed to adhere for 24 hours. The culture media was then replaced with specific inductive media. For adipogenic, osteogenic and chondrogenic differentiation, cells were cultured for two weeks in Adipogenic MSCs Differentiation Bullet Kit, Osteogenic MSCs Differentiation Bullet Kit (Lonza) and NH ChondroDiff Medium (Miltenyi), respectively. Differentiated cell cultures were stained with Oil Red O (Amresco) for adipogenic differentiation, Alizarin Red (Lonza) for osteogenic differentiation or Toluidine Blue (Sigma) for chondrogenic differentiation.

4. Chimeric ligands preparation

4.1. NB260 Chimera Expression and Purification

The chimeras were ceded by Dr. Senyon Choe's group from the Structural Biology Laboratory in the Salk Institute for Biological Studies in La Jolla (California, USA).

To create the NB260 chimera, the mature domains of human Nodal and human BMP2 were divided into six segments. Twelve Nodal/ BMP2 primers corresponding to each segment (Integrated DNA Technologies, Coralville, IA) were combined by an overlapping PCR strategy to produce a full-length PCR fragment of each chimera. To create overlapping regions for PCR, residues Ala77 and Leu95 were substituted in Nodal to valine. The constructs were cloned into pET21A vector (EMD Biosciences, Darmstadt, Germany) and propagated in Nova Blue cells (EMD Biosciences). Final expression constructs were confirmed by DNA sequencing. NB260 chimeras were

expressed in Escherichia coli as inclusion bodies and isolated, purified, and refolded. The refolded ligands were initially purified using Hi-trap heparin sulfate (GE Healthcare) and a sodium chloride gradient. Fractions containing the refolded ligand were submitted to a second round of purification using a C4 reverse phase column (GraceVydac). The protein was eluted using an acetonitrile gradient. The final structure of NB260 was BNNNBN (B: BMP2 segment, N: Nodal segment) (Esquivies et al. 2014).

4.2. AB235 Chimera Expression and Purification

The chimeras were ceded by Dr. Senyon Choe's group from the Structural Biology Laboratory in the Salk Institute for Biological Studies in La Jolla (California, USA).

To create the AB235 chimera, the mature domains of human Activin-βA and human BMP2 were divided into six segments, and primers were designed for each segment. An overlapping PCR strategy was used to mix the various segments together to generate full-length PCR fragment of each chimera. Outer primers for all full-length PCR fragments were constructed to incorporate a 59 NdeI site and a 39 XhoI site for cloning into pET21a expression vector. AB235 chimeras were expressed in E. coli as inclusion bodies. The inclusion bodies were isolated, purified, and refolded. The refolded ligands were purified using Hi-trap Heparin sulfate (GEHealthcare) and reverse phase (GraceVydac) chromatography (Allendorph et al. 2011). The final structure of AB235 was BAAAAA, and mutation to make AB235 Noggin sensitive was introduced: mutating IIKKDIQN from segment 6 to VVLKNYQD using a Quickchange kit. The desired protein sequences were confirmed by DNA sequencing (Peran et al. 2013).

5. Chondrogenic differentiation in cell pellet culture

Cells ($1x10^5$) were grown in 6 well plates in chondrogenic medium and after confluency, at two weeks, the monolayer spontaneously detached from the plastic and took the form of a crumpled paper ball. Control cells grown in incomplete chondrogenic medium did not detach spontaneously from the plastic and, therefore, the monolayer was manually separated using a sterile tip. The emerging pellets were carefully transferred to 15-ml conical tubes and

centrifugated at at 300 x g at 21 °C for 7 minutes. Tubes were incubated with loosened tops at 37 °C and 5% CO_2. Medium was exchanged every other day for the duration of the experiment, and tubes were gently shaken to avoid the adherence of the pellet to the plastic walls.

Incomplete chondrogenic medium was composed by DMEM–high glucose supplemented with 10% FBS, 50 µg/µL of l-ascorbic acid 2-phosphate, 1% penicillin-streptomycin and 1% ITS.

Chondrogenic medium was composed by incomplete chondrogenic medium with fresh 10 ng/ml of chondrogenic inductor (NB260, AB235 or BMP2) added during each medium change every other day.

6. Redifferentiation of dediferentiated chondrocytes in cell pellet culture

Chondrocytes were cultured for 6-7 passages to achieve full dedifferentiation. 300.000 cells/1.5 ml of dedifferentiated chondrocytes were plated into a single well of a six well plate. Control cells were grown in incomplete chondrogenic medium. To induce redifferentiation, cells were cultured in incomplete chondrogenic medium supplemented with 10 ng/ml of AB235 or BMP2. Media was changed every other day and fresh chondrogenic inductor was added to treated pellets during each medium change. After two weeks in culture, control and treated monolayer cells were manually separated using a sterile scraper, transferred to 15 ml conical tubes and then centrifuged at 300 x g at 21 °C for 7 minutes to form a cell pellet. Control and treated pellets were further incubated for four weeks, with media change every other day and fresh AB235 or BMP2 added to treated pellet during each medium change.

7. In vivo assay

In vivo experiments were performed in immunodeficient NOD SCID (NOD.CB17-Prkdcscid/NcrCrl) mice purchased from Charles River (Barcelona, Spain). Cell pellets obtained after 6 weeks of chondrogenic induction were transplanted into the back subcutaneous tissue of mice anesthetized (n=6) by isoflurane inhalation. Animals were maintained in a microventilated cage system with a 12-h light/dark cycle with food and water ad libitum. Mice were manipulated in a laminar air-flow to maintain specific

pathogen-free conditions. Four weeks later, the mice were sacrificed via an overdose injection of anaesthetic, and pellets with new tissue formed around them were excised for further histologic analysis. In vivo assays were carried out in accordance with the approved guidelines of University of Granada following institutional and international standards for animal welfare and experimental procedure. All experimental protocols were approved by the Research Ethics Committee of the University of Granada.

8. Polymer microarray

8.1. Preparation

The polymer microarrays were produced and ceded by Professor Mark Bradley's group from the School of Chemistry at The University of Edinburgh (UK).

Three hundred and eighty members of a pre-synthesised polyurethane (PU) and polyacrylate (PA) library were "spotted" on aminoalkylsilane-treated glass slides, previously coated with agarose to impede unspecific cell adhesion (Pernagallo & Diaz-Mochon 2011). Coating with agarose was achieved by manually dip-coating the slide in agarose Type I-B (1% w/v in deionised water at 65°C), followed by removal of the coating on the bottom of the side by wiping with a clean piece of tissue. Subsequently, the slides were dried overnight at room temperature in a dust-free environment. Polymers for contact printing were prepared by dissolving 10 mg of polymer in 1 ml of the non-volatile solvent N-methyl-2-pyrrolidinone. Polymer microarrays were then fabricated by contact printing (Q-Array Mini microarrayer) with 32 aQu solid pins (K2785, Genetix) using the polymer solutions placed in polypropylene 384-well microplates (X7020, Genetix). The 380 members of the polymer libraries were printed following a four-replicate pattern with 1 single field of 32×48 spots containing 4 control (emptied) areas. Printing conditions were as follows: 5 stampings per spot, 200 m sinking time, and 10 ms stamping time. The typical spot size was 300–320 µmin diameter with a pitch distance of 560 µm (y-axis) and 750 µm (x-axis), allowing up to 1520 features to be printed on a standard 25×75 mm slide. Once printed, the slides

were dried under vacuum (12 h at 42 °C/200 mbar) and sterilised in abio-safety cabinet by exposure to UV irradiation for 20 min prior to use.

8.2. Cell culture

For polymer library screening, suspensions of cell populations in 5 ml of media were plated (3×10^5 cells/well) onto two identical polymer microarrays containing 380 polymers (PUs and PAs).

Articular chondrocytes were grown in DMEM–high glucose (Sigma) supplemented with 10% FBS (Sigma), 50 μg/μL of l-ascorbic acid 2-phosphate (Sigma), 1% penicillin-streptomycin (Sigma) and 1% ITS (Gibco). ASCs and IFPSCs, were grown in DMEM–high glucose (Sigma) supplemented with 10% FBS and in the presence of 1% penicillin–streptomycin (Sigma). To direct chondrogenic differentiation, IFPSCs and ASCs were cultured in chondrogenic medium consisting in DMEM–high glucose (Sigma) supplemented with 10% FBS, 1% penicillin–streptomycin, 10 ng/ml of the AB235 chimeric ligand, 1% ITS (Gibco) and 50 μg/μL of l-ascorbic acid 2-phosphate (Sigma). ASCs and IFPSCs were cultured in chondrogenic media for 2 weeks before the experiments.

9. Polymer coating of well plates.

9.1. Preparation

The preparation of the polymer powder was performed by Sesha Venkateswaran, PhD student in the group of Proff. Bradley in the School of Chemistry at The University of Edinburgh (UK).

For large-scale analysis, polymer coating of 12 well plates were prepared by incubating 250 μL of each polymer solutions (2.0% w/v in acetic acid) for 30 min at 4ºC under gentle shaking and left to air dry overnight in the hood at RT. The coated wells were

then irradiated with UV light for 30 min and washes with PBS two times (5 min per wash) prior to cellular studies.

9.2. Cell culture

For a large-scale analysis of the hit polymers, cells were seeded at $3x10^4$ cells per well of the polymer-coated 12 well plate. After 10 days in culture, cells were fixed with 4% paraformaldehyde for 20 min at room temperature (RT).

Articular chondrocytes, ASCs, IFPSCs, AB235-treated ASCs and AB235-treated IFPSCs were grown as previously described in 8.1 section.

10. Hydrogel synthesis

9.1. Preparation

The preparation of the hydrogel was performed by Sesha Venkateswaran, PhD student in the group of Prof. Bradley.

Combinations of monomers for PA204 and PA391 were dissolved in 1-methyl-2-pyrrolidinone (NMP) and solutions of tetramethylethylene diamine (TEMED). Then, PEGDA, PEG or PETA were added sequentially as crosslinkers (see Table). The solutions were mix 1 min, and after that the hydrogel polymer synthesis was achieved by adding the redox initiator (ammonium persulfate (APS). Finally, the reaction solution was kept in a 37 ºC incubator overnight. The hydrogels were washed with ethanol three times (3× 30 min) and with PBS four times to remove unreacted material (3×30 min and 1×overnight washes) and stored with water at RT. In order to obtain lyophilized scaffolds, hydrogels were frozen on dry ice for 5 min, transferred at −80 °C overnight and then dried in the following conditions: 1 mbar and −45°C.

Unlyophilized and lyophilized hydrogels were cut into 2 mm discs. Unlyophilized hydrogels were sterilized with 70% (v/v) ethanol 30 min and then rinsed three times in

PBS, whereas surfaces of unlyophilized and lyophilized were treated with ultraviolet (UV) light for 15 min.

Table 1. Hydrogels of PA204 and PA391 polymers.

POLYMER	PEGDA	PEG	PETA
PA204-1	X		
PA204-2	X	X	
PA204-2A	X	2X	
PA391-1	X		
PA391-2	X		X
PA391-2A	X	X	X

10.2. Cell culture

Hydrogels were maintained 4 hours in medium, and after that, a 2x105cells/100 µL aliquot of suspended chondrocytes solution was pipetted onto each specimen and incubated for 4 h at 37ºC to allow the cells to attach. Afterwards, the cell-seeded scaffolds were transferred into new 24-well culture plates with 1mL of medium. All samples were further incubated under 5% CO_2 atmosphere at 37^{o}C for 48 hours or 21 days. The culture medium was regularly replaced every 2 days.

11. Polymer-coated PCL scaffolds

11. 1. Preparation

The preparation of the polymer-coated PCL scaffolds was performed by Sesha Venkateswaran, PhD student in the group of Prof. Bradley.

PCL scaffolds (Sigma) were coated with PA-204 and PA-391 polymer solution (2.0% w/v in acetic acid) and left to air dry overnight in the hood at RT. Uncoated and coated-PCL scaffolds were placed into 12 wells and each surface sterilized by UV irradiation for 15 min.

11.2. Cell culture

The seeding was performed by applying the chondrocytes suspension, with a concentration of $2x10^5$ cells in 200 µL of medium onto each scaffold. After 4 h at 37ºC, 2 mL of medium was added. All samples were further incubated under 5% CO_2 atmosphere at 37^oC for 48 hours or 21 days. The culture medium was regularly replaced every 2 days.

12. Molecular analysis

12.1. RNA extraction

Total cellular RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's recommendations. Briefly, add 1 ml of Trizol by sample and homogenizer by pipetting or vortex and then add 0.2 ml of chloroform and leave 15 minutes at RT to ensure complete dissociation of nucleoprotein complexes. Centrifuge the resulting mixture at 12,000 × g for 15 minutes at 4 °C. Transfer the supernatant (colorless upper aqueous phase) to a fresh tube and add 0.5 ml of 2-propanol, allo the sample to stand for 10 minutes at RT and centrifuge at 12,000 x g for 10 minutes at 4°C-The RNA precipitate will form a pellet on the side and bottom of the tube,remove the supernatant and wash the RNA pellet by adding a minimun of 1 ml of 75% ethanol, vortex the sample and then centrifuge at 7,500 × g for 5 minutes at 4 °C. Briefly dry the RNA pellet for 5–10 minutes by air-drying and add 20 microlitres of free-RNAse water.

12.2. Reverse transcription–polymerase chain reaction

Total RNA was reverse transcribed using the Reverse Transcription System kit (Promega, Madison, WI, USA). Briefly, incubate 1µg of RNA in 10 µl at 70°C for 10 minutes. Then, add the following reagents: MgCl2, 25mM (4µl) Reverse Transcription 10X Buffer (2µl) dNTP Mixture 10mM (2µl) Recombinant RNasin Ribonuclease Inhibitor(0.5µl) AMV Reverse Transcriptase (High Conc.) (15u) and Random Primers (0.5 µg). Incubate the reaction at room temperature for 10 minutes, then incubate at 42°C for 15 minutes, heat the sample at 95°C for 5 minutes, then incubate at 0–5°C for 5 minutes.

12.3. Real-time PCR analysis

Real-time PCR was performed using the SYBR-Green PCR Master mix (Promega) according to the manufacturer's recommendations. PCR reactions were performed as follows: an initial denaturation at 95°C for 2 min, 40 cycles of 95°C for 5 s and 60°C for 30 s, and a final cycle of dissociation of 60 – 95 °C. The gene expression levels were normalized to corresponding GAPDH values and are shown as fold change relative to the value of the control sample. All the samples were done in triplicate for each gene.

Table 2. Sequences of the primers used in the RT-PCR reactions

Gene	Forward	Reverse
Col I	ATGGATGAGGAAACTGGCAACT	GCCATCGACAAGAACAGTGTAAGT
Col II	GAGACAGCATGACGCCGAG	GCGGATGCTCTCAATCTGGT
Col X	GCCCACTACCCAACAC	TGGTTTCCCTACAGCTGA
COMP	AACACGGTCACGGATGACGACTATG	CACAGAGCGTTCCGCAGCTGTTC
ACAN	AGGATGGCTTCCACCAGTGC	TGCGTAAAAGACCTCACCCTCC
Sox9	ACTCCGAGACGTGGACATC	TGTAGGTGACCTGGCCGTG
GADPH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

13. Histological analysis

3D culture system (pellets, hydrogels and polymer-coated PCL) were immersed in 4% paraformaldehyde in 0.1 M PBS for 4 hours at 4°C, washed in 0.1M PBS and embedded in paraffin in an automatic tissue processor (TP1020, Leica, Germany). The paraffin blocks were cut into 4 µm sections for staining. 1. Deparaffinization and rehydration of slides: a) Tissue sections were deparaffinized in Xylene, 4 changes of 5 min per change. b) Hydrated in 100% ethanol, 1 change of 3 min. c) Hydrated in 95% ethanol, 1 change of 3 min. d) Washed in tap water for 3 min. After the staining, the slides were dehydrated and cleaned: a) Dehydrated in 95% ethanol, 2 changes of 2 min per change. b) Dehydrated in 100% ethanol, 1 change of 2 min. c) After the tissue sections were cleared in Xylene, 2 changes of 2 min per change. Finally, tissue sections were mounted and observed under microscope.

13.1. Hematoxilyn-Eosin staining

The following protocol was used:

1. Stain in hematoxylin (Panreac) for 3 minutes. Always filter before each use to remove oxidized particles
2. Rinse in running tap water for 10 minutes
3. Counterstain in Aqueous Eosin 1 min: 1 g of eosin (Panreac) + 100 ml of distillated water + 1 ml of acetic acid (add in the moment of the staining)
4. Wash gently in top water, approximately 4 changes or until excess dye stops leaching out of tissue for 10 minutes

13.2. Toluidine Blue staining

The following protocol was used:

1. Stain with 0,04% Toluidine O Blue solution for 20 min.

   a. 0.1M Sodium Acetate Buffer: 13.6 g of Sodium Acetate anhydrous + 1 L of deionized water. Adjust final pH at 4 using Glacial acetic acid. Store at RT or 4ºC for longer storage
   b. 0.4% Toluidine O Blue Solution: 0.4 g of Toluidine Blue O (Sigma) + 100 ml of 0.1M Sodium Acetate Buffer
2. Rinse gently with 3 changes of deionized water, (30 sec each)

For Toulidine blue stain in monolayer, cells were washed twice in PBS and fixed with 4% paraformaldehyde for 20 min at RT. Cells were stained in Toulidine Blue (Sigma) solution (0.1% in distillated water) for 5 minutes at RT, and rinsed with deionized water until the excess stain washed away.

13.3. Alcian Blue staining

The following protocol was used:

1. Glacial acetic acid 3% for 3 minutes
2. Stain in alcian blue solution for 30 minutes: 1 g of Alcian Blue (Panreac) + 100 of Acetic acid at 3%. Adjust final pH at 2.5 using Glacial acetic acid
3. Wash in running tap water for 2 minutes
4. Rinse in distilled water
5. Stain Harris Hematoxilina solution for 1 minute
6. Wash in running tap water for 1 minute

13.4. Masson´s Trichrome staining

The following protocol was used:

1. Bouin´s solution during 24h
   a. Picric solution: 2 g of Picric acid (Fluka) + 100 ml of distillated water

   b. 75 ml of Picric solution + 25 ml of formaldehyde (37 – 40%) + 5 ml of glacial acetic acid
2. Rinse running tap water for 5-10 minutes to remove the yellow color.
3. Stain in Weigert's iron hematoxylin working solution for 10 minutes.
   a. Stock Solution A: 1 g of Hematoxylin (Panreac) + 100 ml of 95% alcohol. Not expired
   b. Stock Solution B: 4 ml of 29% Ferric chloride in water (Merk) + 95 ml of distillated water + 1 ml of Hydrochloric acid concentrated. Not expired
   c. Mix equal parts of stock solution A and B. This working solution is stable for about 4 weeks.
4. Rinse in running warm tap water for 10 minutes
5. Stain in Biebrich scarlet-acid fuchsin solution for 3 minutes (Add glacial acetic acid). Solution can be saved for future use.
6. Wash in distilled water.
7. Differentiate in phosphomolybdic-phosphotungstic acid solution for 15 minutes or until collagen is not red: 5 g of Phosphomolybdic acid x-hydrate (Panreac) + 5 g of Phosphotungstic acid hydrate PA (Panreac) + 200 ml of distilled water. Not expire.
8. Transfer sections directly (without rinse) to 0.02% light green solution and stain for 7-10 minutes: 2 g of Light green SF yellowish (Merck) + 98 ml of distilled water + 1 ml of glacial acetic acid (add in the moment of the staining)
9. Wash in distilled water.

13.5. Alizarin Red staining

The following protocol was used:

For Alizarin Red stain in monolayer, cells were washed twice in PBS and fixed with 4% paraformaldehyde for 20 min at RT. Cells were stained in Alizarin Red (Sigma) solution (2 g of Alizarin Red + in 100 ml of deionized water, and pH was adjusted to 4.3 with ammonium hydroxide) for 15 minutes at RT, and rinsed with deionized water until the excess stain washed away.

14. Immunocytochemistry

For immunofluorescence staining, cells and 3D culture system (pellets, hydrogels and polymer-coated PCL) were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at RT. For intracellular staining, cells were permeabilized with 0.1% Triton X-100 for 15 min. Then, cells were blocked in 2% blocking buffer solution (Roche) for 1 h at RT. Primary antibodies were incubated overnight at 4ºC, and secondary antibodies were incubated at room temperature for 2 hours. Afterwards, they were washed three times in PBS and the slides were mounted using Vectashield containing DAPI. Photographs were taken with a Leica DM 5500B (Solms, Germany) fluorescent microscope, software Meta Systems Isis, or confocal microscopy (Nikon Eclipse Ti-E A1, USA) and analyzed using NIS-Elements software.

Primary antibodies used for immunocytochemistry were: i) anti-collagen type I (Santa Cruz Biotechnology), anti-collage type II (Santa Cruz Biotechnology), anti-collagen type X (Abcam), anti-Sox9 (Millipore) and anti-Aggrecan (Sigma). All antibodies were diluted 1:100.

Secondary antibodies used were: Fluorescein Iso-thiocyanate (FITC) or Phycoerythrin (PE) (Santa Cruz Biotechnology) diluted 1:200.

15. Quantitative image analysis

Quantitative image analysis was performed using ImageJ software (v1.43, NIH). Area, integrated density and mean staining were measured, along with several adjacent background readings. The relative staining was calculated using = integrated density – (selected area × mean staining of background readings) (Jensen 2013) (McCloy et al. 2014). All samples were measured in triplicate and different sections of the pellets were analysed.

16. Scanning for cell binding in polymer microarrays

The analysis of the polymer microarrays was performed by Sesha Venkateswaran PhD student in the group of Proff. Bradley in the School of Chemistry at The University of Edinburgh, Dr. Salvatore Pernagallo that belong to the company DestiNA Genomics S.L., and Dr. Juan José Díaz Mochón member of the University of Granada and Junta de Andalucía Centre for Genomics and Oncological Research (GENYO).

After 3 days, cells were washed two times with PBS and labeled with Cell Tracker Green/CMFDA (Invitrogen, USA) following the manufacturer instructions. Then, cells were fixed with 4% paraformaldehyde in PBS for 20 min at RT and stained with DAPI. Image capture and analyses were carried out using a high content screening platform (Nikon 50i fluorescence microscope with an X-Y-Z stage), equipped with Pathfinder TM software (IMSTAR S.A., France), using an x20 objective. Cell numbers on each polymer member was determined using fluorescent (DAPI, FITC-like bandpass filters) channels with automated scanning of the polymer spots.

17. Live/dead fluorescence staining

The viability and distribution of chondrocytes inside the 3D composite hydrogels and uncoated and polymer-coated PCL scaffolds were detected by live and dead staining using Cell Tracker Green/CMFDA (CTG) and propidium iodide (PI). Briefly, samples were washed two times with PBS and incubated for 15 min at 37°C with CTG and 25 µg/mL PI. Afterwards, samples were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at RT and stained with DAPI. Living and dead cells were photographed by confocal microscopy (Nikon Eclipse Ti-E A1, USA) and analyzed using NIS-Elements software.

18. Glycosaminoglycans content analysis

Total glycosaminoglycans (GAGs) content on the cell-seeded hydrogels was determined using 1,9-dimethylmethylene blue (DMB) dye solution (Sigma). Briefly, after the 7, 14, and 21-day culture period, the cell-seeded hydrogels were digested with 1 mL papain solution (5mM cysteine, 250 µg/mL papain, 200m M NaH 2PO 4 and 5nM M EDTA) at 60ºC for 16 h. The cell solution was centrifuge at 13.000 rpm for 10 minutes at 4ºC and a DMB solution was applied to the supernatant of each sample. Absorbance was measured at 520 nm, and compared to a chondroitin sulfate solution standard (Sigma).

19. Environmental scanning electron microscope (ESEM) analysis

After 21 days of culture, samples were fixed in 2% glutaraldehyde during 4 h at 4ºC and then were rinsed several times with sodium cacodylate. The observations started at an initial water vapor pressure of 5.7 tors. At this pressure, a liquid water phase was present in the sample (100% RH). Then, vapor pressure was decreasing slowly until the surfaces of the samples were visible (2 and 2.4 tors). Accelerating voltages varied between 10 and 15 kV, we obtained a good image resolution using small beam current (spot size 2) and a working distance of 6.1 e 7.5 mm. The environmental scanning electron microscope (ESEM) used in this work was a Quanta 400 (FEI) located at the Centro de Instrumentación Científica of the University of Granada.

20. Statistics analysis

All graphed data represent the mean +/-SD from at least three experiments. Differences between treatments were tested using the two tailed Student´s T test. Assumptions of Student´s T test (homocedasticity and normality) were tested and assured by using transformed data sets [log(dependent variable value +1)] when necessary. P-values <0.01 (**, ##, ††) and <0.05 (*, #, †) were considered statistically significant in all cases.

RESULTS AND DISCUSSION

CHAPTER I

Chondrogenic differentiation of adipose-derived stem cells

Results

1. Cell isolation and characterization of IFPSCs and ASCs

In order to compare the chondrogenic potential of adipose derived stem cells isolated from two different sources we obtained adipose tissue from the infrapatellar fat pad of osteoarthritis patients and from patients undergoing a liposuction surgery for aesthetic issues.

Adipose derived stem cells were isolated by enzymatic collagenase digestion and named as IFPSCs for cells obtained from the infrapatellar fat pad and ASCs for cells isolated from lipoaspirates. Following the recommendation of the International Society for Cellular Therapy (ISCT), IFPSCs and ASCs were characterized according to the minimal criteria to define human mesenchymal stem cells (hMSC).

Freshly isolated IFPSCs and ASCs presented a typical spindle shape fibroblastic morphology when were cultured on plastic surfaces under standard culture conditions, as can be appreciate in Figure 5A and Figure 6A. At passage 4 cells were tested for specific surface hMSC markers (CD73, CD90, CD105), and endothelial and hematopoietic markers (CD133, CD34 CD45) expression. Fluorescence-activated cell sorting analysis (FACS) in ASCs showed a high expression of CD73 (100%), CD90 (98%) and CD105 (98%). Similarly, IFPSCs presented a high expression of CD73 (100%), CD90 (99.6%) and CD105 (99.7%). On the other hand, ASCs showed a low expression of endothelial and hematopoietic markers: CD45 (1%), CD133 (1.2%) and CD34 (3.5%). Also, IFPSCs revealed similar values for these markers: CD45 (0.4%), CD133 (0.6%) and CD34 (3.2%) [Fig. 5B and Fig. -6B].

We further tested the plasticity potential of the isolated cells checking their differentiation capacity towards chondrocytes, adipocytes and osteoblasts. IFPSCs and ASCs were cultured under standard *in vitro* culture-differentiating conditions during 2 weeks. The acquisition of an adipocyte-like phenotype was confirmed by the deposits of lipid stained with Oil Red. Osteogenic differentiation was confirmed by calcific deposition using Alizarin Red staining. Finally, chondrogenic differentiation was assessed by the presence of proteoglycans stained with Toluidine Blue. Cells islated

from both sources are capable of differentiating into other mesenchymal tissue types, including adipocytes, chondrocytes, myocytes and osteoblasts [Fig. 5C and Fig. 6C]

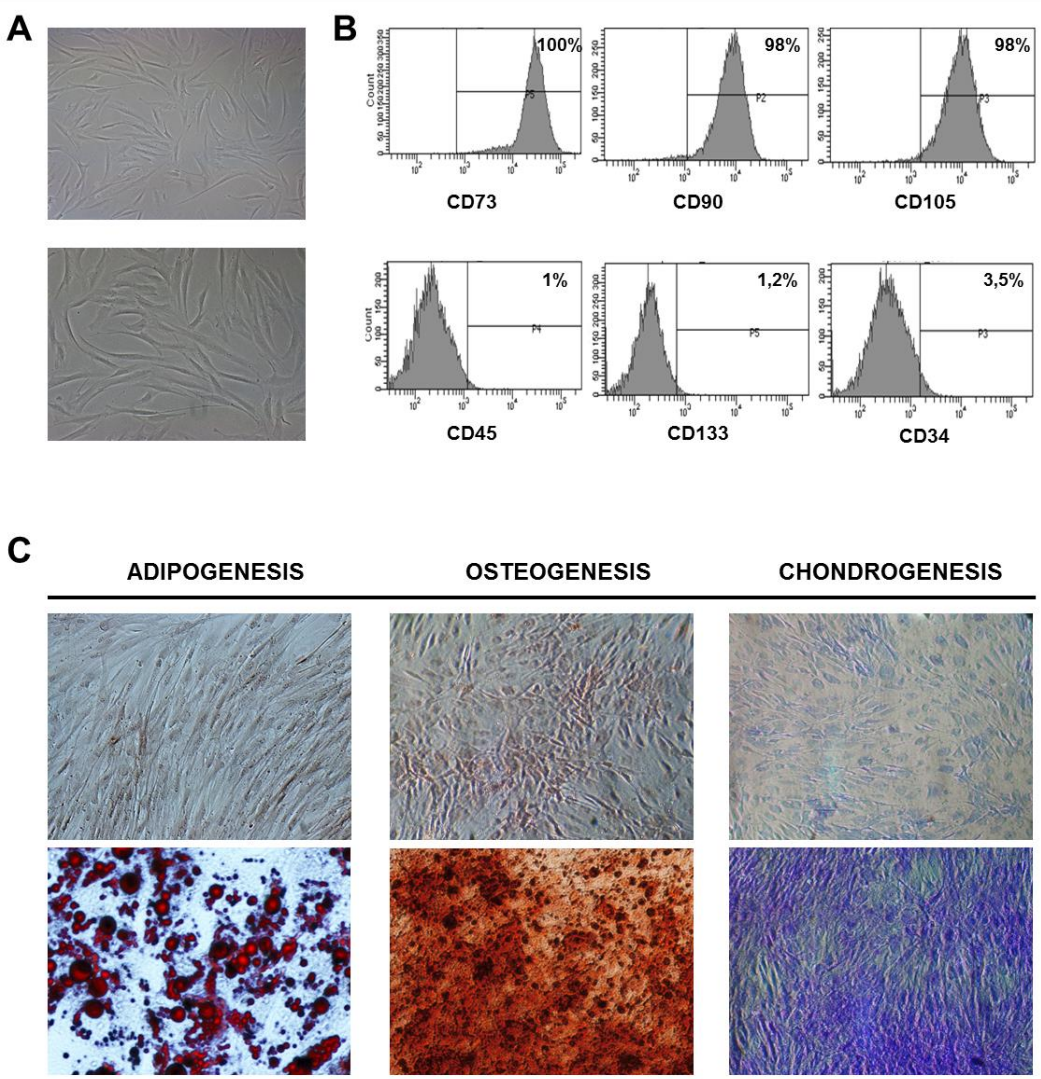

Fig. 5. Phenotypic characterization and differentiation potential of ASCs. **(A)** Phase-contrast light microscopy of cultured ASCs at passage 4. Original magnification: 10X and 20X. **(B)** FACS characterization demonstrated a positive expression of the surface markers CD73 (100%), CD90 (98%), CD105 (98%) and a negative expression for CD45 (1%), CD133 (1.2%) and CD34 (3.5%). **(C)** The differentiation potential of ASCs towards adipogenic, osteogenic and chondrogenic lineage was confirmed by Oil Red O, Alizarin Red S and Toluidine Blue staining, respectively. Upper pictures show negative controls, cells cultured in normal medium for 2 weeks and then histochemically stained. Original magnification: 20x.

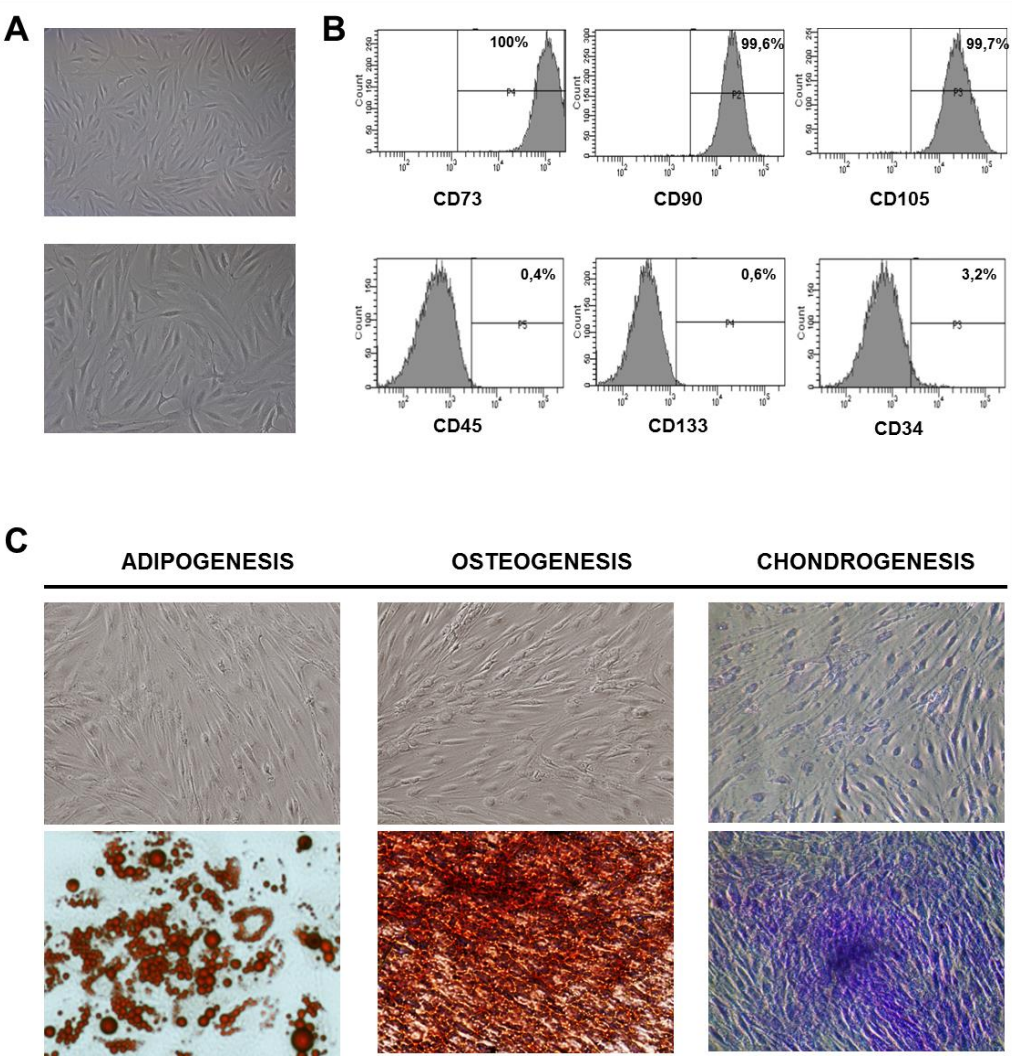

Fig. 6. Phenotypic characterization and differentiation potential of IFPSCs. **(A)** Phase-contrast light microscopy of cultured IFPSCs at passage 4. Original magnification: 10X and 20X. **(B)** FACS characterization demonstrated a positive expression of the surface markers CD73 (100%), CD90 (100%), CD105 (100%) and a negative expression for CD45 (0.4%), CD133 (0.6%) and CD34 (3.2%). **(C)** The differentiation potential of IFPSCs towards adipogenic, osteogenic and chondrogenic lineage was confirmed by Oil Red O, Alizarin Red and Toluidine Blue staining, respectively. Upper pictures show negative controls, cells cultured in normal medium for 2 weeks and then histochemically stained. Original magnification: 20x.

2. Chondrogenic differentiation potential of AB235 and NB260

The objective of our study was to compare the chondrogenic potential of Nodal/BMP2 and Activin/BMP2 chimeric ligands (NB260 and AB235, respectively) in adipose derived stem cells isolated from the two different tissue sources.

IFPSCs and ASCs were cultured in a pellet system as described previously (Peran et al. 2013). Briefly, cells were grown in monolayer for two weeks and, at this point, monolayers of cells treated with NB260 and AB235 were markedly more confluent than monolayer of control non-treated cells. NB260 and AB235 confluent monolayers spontaneously detached from the plastic acquiring the appearance of a crumpled paper ball. Detached-monolayers were then transferred to conic tubes and cultured under pellet system conditions for four weeks. As untreated cells did not detach spontaneously from the plastic the monolayer was manually separated using a sterile tip. Cell pellets were cultured for six weeks in media containing NB260, AB235 or without treatment and processed for histological, immunofluorescence and real-time PCR analysis. BMP2 was used as positive control.

2.1. Cell pellets analysis: size and extracellular matrix formation

In order to determine the chondrogenic potential of the chimeric ligands and their possible role in inducing ECM production, the size of the cell pellets were measured and pellet sections were processed for histological and immunofluorescence analysis.

Results showed that cell pellets treated with the chimeric ligands AB235 and NB260 showed higher size in comparison with untreated control pellets in both types of cells. Cells pellets grown with BMP2 supplementation (as positive control for chondrogenesis) showed an increase of size when compared with control pellets that was similar than those treated with NB260 and lower than pellets treated with AB235 [Fig. 7A and Fig. 8A].

Cell pellets that were induced under supplementation condition showed a shiny and compact appearance similar to native cartilage. On the other hand, untreated control pellets showed less consistent appearance, in special IFPSCs pellets resembling a cellular cluster without a dense ECM.

Interestingly, for both cell types cell pellets cultured in AB235-containing medium were larger relative to NB260 and BMP2 induced pellets. In addition, ASCs pellets cultured under the three chondrogenic conditions, AB235, NB260 and BMP2, were larger than IFPSCs pellets cultured under the same circumstances, whereas untreated control pellets of both cell types showed similar size [Fig. 7A and Fig. 8A].

NB260, AB235 and BMP2 treated pellets and control non-treated cell pellets were processed for histological analyses. In pellets cultured with NB260, AB235 and BMP2 Hematoxilyn-Eosin staining revealed a complex internal structure similar to native cartilage tissue, with cells (dark purple) isolated in lacunae surrounded by dense ECM (pink). Alcian Blue and Toluidine Blue staining in chimeric and BMP2 treated pellets showed the presence of proteoglycans (blue and purple, respectively) in the ECM, and Masson-Trichrome staining revealed the presence of the other majority component of the ECM, collagens fibres (green), very similar to native cartilage tissue. However, in untreated control pellets the presence of proteoglycans and collagens were less evident [Fig. 7B and Fig. 8B]. ImageJ was used for quantitative image analysis, and the staining of pellets cultured with NB260, AB235 and BMP2 was significantly higher ($p<0.01$) when compared with untreated control pellets. Moreover, the staining between the treatments was very similar, without significant differences [Fig. 9A].

Immunofluorescence analysis for collagens expression showed higher expression of Col II in pellets cultured with NB260 and AB235-containing medium, and reduced expression of Col I, that was restricted to the edges of the pellets. BMP2 showed different pattern depends of the type of cells, in IFPSCs pellets the expression of Col I and Col II were the same that in pellets treated with AB235 and NB260, whereas in ASCs pellets treated with BMP2 presented majority expression of Col I and reduced expression of Col II. In ASCs untreated control pellets the expression of Col II was less

and diffuse that in treated pellets. Further, in IFPSCs untreated control pellets not expression of Col II was detected, so that 3D culture system can induce a minimal chondogenesis in ASCs cultured without cytokines. Likewise, Sox9 expression was higher in treated pellets than in untreated control pellets [Fig. 7C and Fig. 8C].

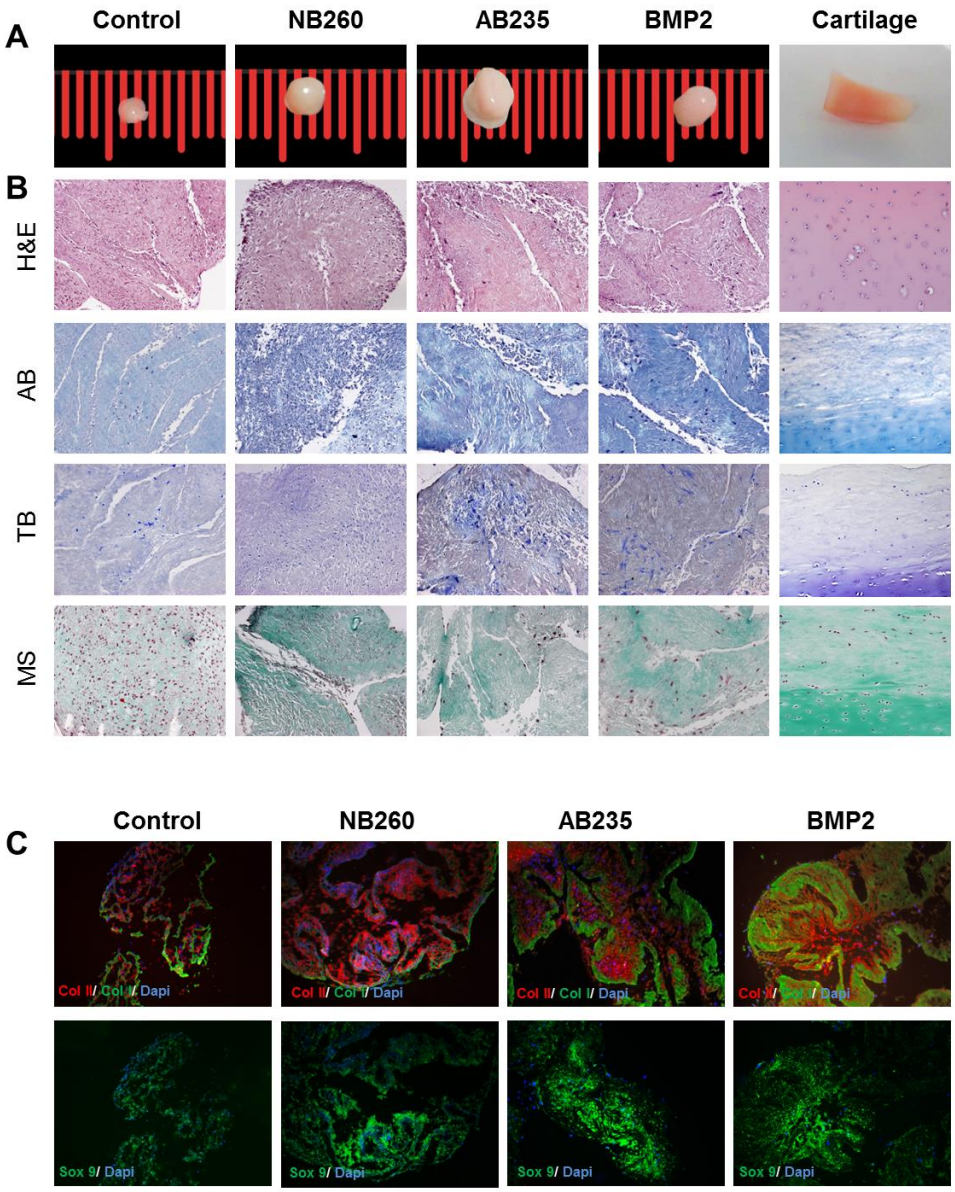

Fig. 7. **Chondrogenic induction of ASCs with NB260 and AB235-chimeric ligands**. **(A)** Representative imagines of ASCs cultured in a pellet system for 6 weeks, treated with 10 ng/ml of the chimeric ligand NB60 or AB235. Untreated pellets, BMP2 pellets and native cartilage tissue were used as control. **(B)** Histological staining of pellets sections with Hematoxilin-Eosin (H&E), Alcian blue (AB), Toluidine blue (TB) and Masson's Trichrome (MS) showed the typical

morphology of the native cartilage and the presence of collagens and GAGs in the ECM. **(C)** Immunofluorescence staining of pellets sections with Col II (red channel), Col I (green channel) and Sox9 (green channel) antibodies demonstrate an increase of chondrogenic proteins expression and feature spatial distribution induced by the NB260 and AB235 ligands. Original magnification: 20x for all panels.

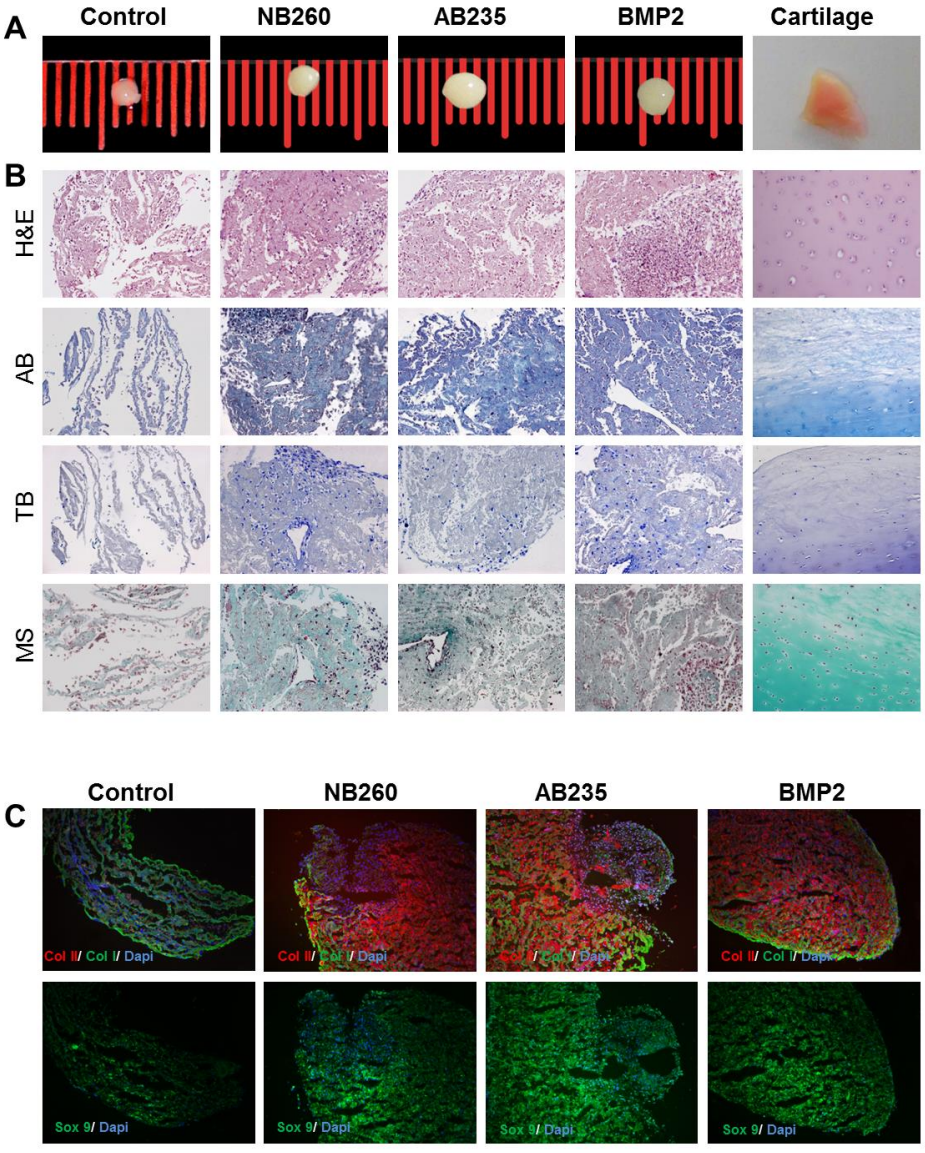

Fig. 8. Chondrogenic induction of IFPSCs with NB260 and AB235-chimeric ligands. **(A)** Representative imagines of IFPSCs cultured in a pellet system for 6 weeks, treated with 10 ng/ml of the chimeric ligand NB60 or AB235. Untreated pellets, BMP2 pellets and native cartilage tissue were used as control. **(B)** Histological staining of pellets sections with Hematoxilin-Eosin (H&E), Alcian blue (AB), Toluidine blue (TB) and Masson's Trichrome (MS) showed the typical morphology of the native cartilage and the presence of collagens and GAGs

in the ECM. **(C)** Immunofluorescence staining of pellets sections with Col II (red channel), Col I (green channel) and Sox9 (green channel) antibodies demonstrate an increase of chondrogenic proteins expression and feature spatial distribution induced by the NB260 and AB235 ligands. Original magnification: 20x for all panels.

In addition, ImageJ was used for quantitative image analysis. Control ASCs pellets showed lower significant expression of Col II and Sox9 than treated pellets ($p < 0.01$), as well as lower expression in Col I in comparison with AB235 and BMP2-cultured pellets ($p<0.01$), but higher related to NB260 ($p<0.01$). When different treatments of ASCs pellets were compared, pellets cultured with NB260 showed less expression of Col I and Sox9 ($p<0.01$), and significantly higher of Col II ($p<0.05$) that AB235-cultured pellets, while control BMP2 treated pellets showed more hypertrophic phenotype that pellets cultured with chimeric ligands, with high expression of Col I ($p<0.01$), and lower expression of Col II ($p <0.01$). In contrast, in IFPSCs pellets the ECM matrix displayed the same composition between the different treatments (NB260, AB235 and BMP2) without significant differences, but with high expression of chondrogenic markers, Col II ($p <0.01$) and Sox9 ($p <0.01$), in comparison with untreated control pellets [Fig. 9B].

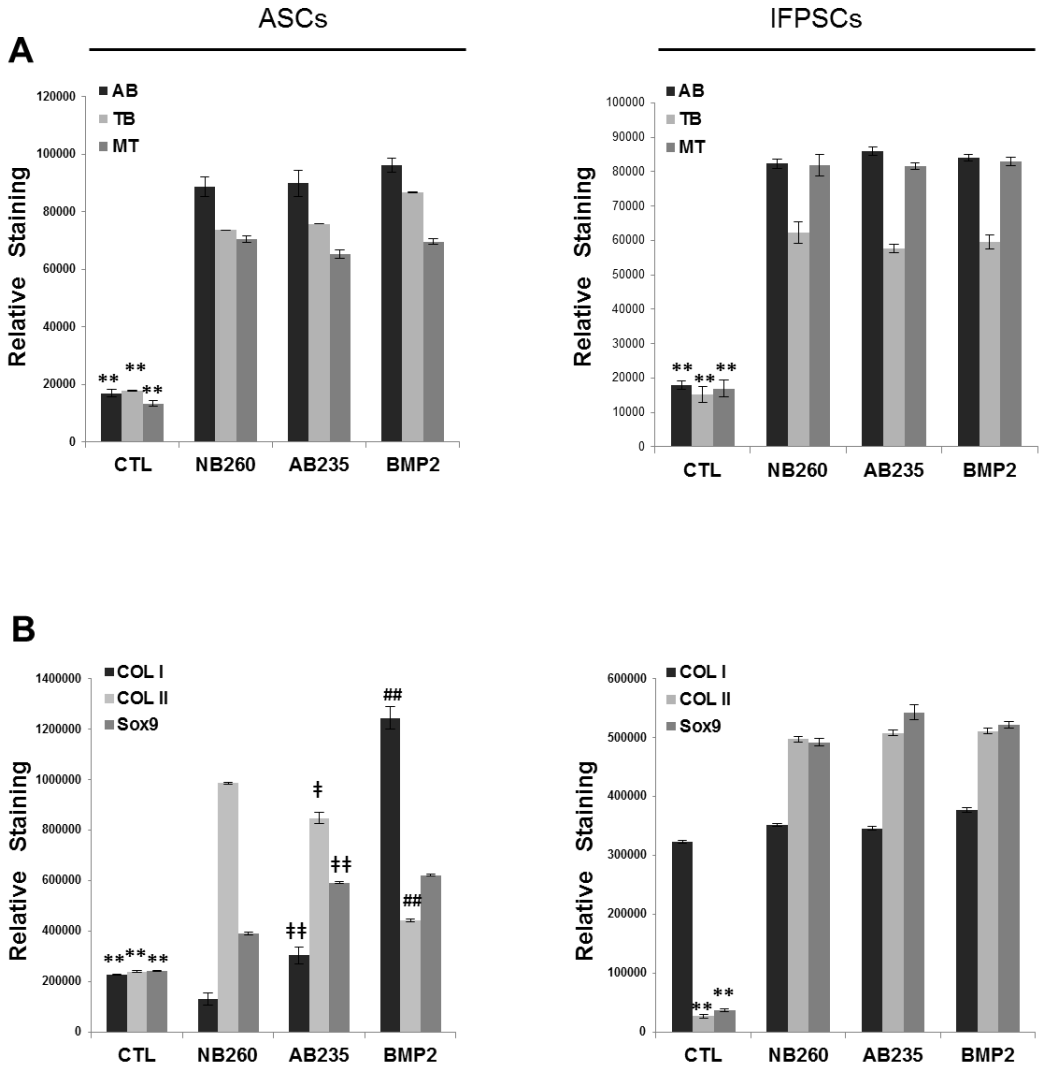

Fig. 9. **Quantitative image analysis. (A)** Graphical representation of histological and **(B)** immunofluorescence quantification staining in control and NB260, AB235 or BMP2-treated pellet sections. MT: Masson-Trichrome; AB: Alcian Blue; TB: Toluidine blue. Statistical significant differences were found when compared untreated control pellets and treated pellets (** $p<0.01$) in both cell types, and in ASCs pellets when compared with NB260 and AB235 (## $p<0.01$), and BMP2 respect to chimeras (†$p<0.05$, ††$p<0.01$).

2.2. Real-time PCR analysis

Real-time PCR (qPCR) analysis was performed to quantify expression of genes related to chondrogenic differentiation after 6 weeks of treatment with the chimeric ligands.

We first compared which cell type was more amenable to treatment with the chimeric ligands. ASCs pellets cultured with NB260 chimera showed higher expression for

chondrogenic gene-markers Col I ($p < 0.05$), ACAN, Sox9 and COMP ($p < 0.01$) that IPSCs pellets. Furthermore, the expression of hypertrophic markers Col I and Col X ($p < 0.01$) were higher in ASCs pellets. AB235 and BMP2 treatment displayed similar results with enhanced Col I, ACAN, Sox9 and COMP ($p < 0.01$), as well as Col I and Col X ($p < 0.01$) in ASCs pellets in comparison with IFPSCs pellets. Definitely, ASCs showed higher expression of chondrogenic genes and hypertrophic genes than IFPSCs [Fig.10].

Second, we compared which of the chimeric ligand was more effective in inducing chondrogenesis comparing chodrogenic markers expression in cells treated with the chimeric ligands versus cells treated with the positive control BMP2. In addition, the chondrogenic effect of each AB235 or NB260 was compared between them. Real-time PCR determinations showed that in IFCPSCs pellets treated with AB235 the expression of Col II, ACAN, Col I and Col X ($p < 0.05$) was lower than in NB260 treated pellets, but the expression of Sox9 and COMP was higher ($p < 0.01$). Moreover, in IFPSCs pellets cultured with AB235 the expression of Col II, Col I and Col X was similar to the one found for BMP2 treated pellets, without significant differences, but the expression of other chondrogenic markers (Sox9, ACAN and COMP) was significant higher in AB235 pellets ($p < 0.01$) than BMP2 pellets. On the other hand, in IFPSCs pellets cultured with NB260 incremented the expression of all the genes in comparison with BMP2 treated pellets ($p < 0.01$), except Col II that was similar [Fig. 11]. All these data indicate that in IFPSCs, chimeric ligands possess more chondrogenic differentiation potential than BMP2.

In ASCs pellets, AB235-containing medium incremented Col II, ACAN, Sox9 and Col I expression ($p < 0.01$, $p < 0.05$), and decreased the expression of COMP and Col X in comparison with NB260 treatment ($p < 0.01$, $p < 0.05$). Moreover, when compared AB235 treatment with BMP2, AB235 chimeric ligand incremented Col II and Sox9 expression ($p < 0.01$) and decreased COMP and Col X ($p < 0.01$), whereas, the expression of chondrogenic markers and hypertrophic markers were significant lower in NB260-containing medium in comparison with BMP2 ($p < 0.01$, $p < 0.05$) [Fig. 11].

In summary, ASCs pellets cultured with NB260 and AB325-containing medium showed higher expression of chondrogenic genes (Col II, Sox 9, ACAN and COMP) and hypertrophic genes (Col I and Col X) that IFPSCs pellets ($p<0.01$) [Fig. 10]. In IFPSCs and ASCs pellets, in general, AB235 treatment showed an increment in expression of some chondrogenic markers and a decrease in hypertrophic markers expression when compared with NB260 treatment [Fig. 11].

All together, histological, immunofluorescence and qPCR data indicate that NB260 and NB250 chimeras can induce chondrogenic differentiation in adipose-derived stem cells, in special in ASCs, even more efficiently that typical BMP2 chondrogenic inductor.

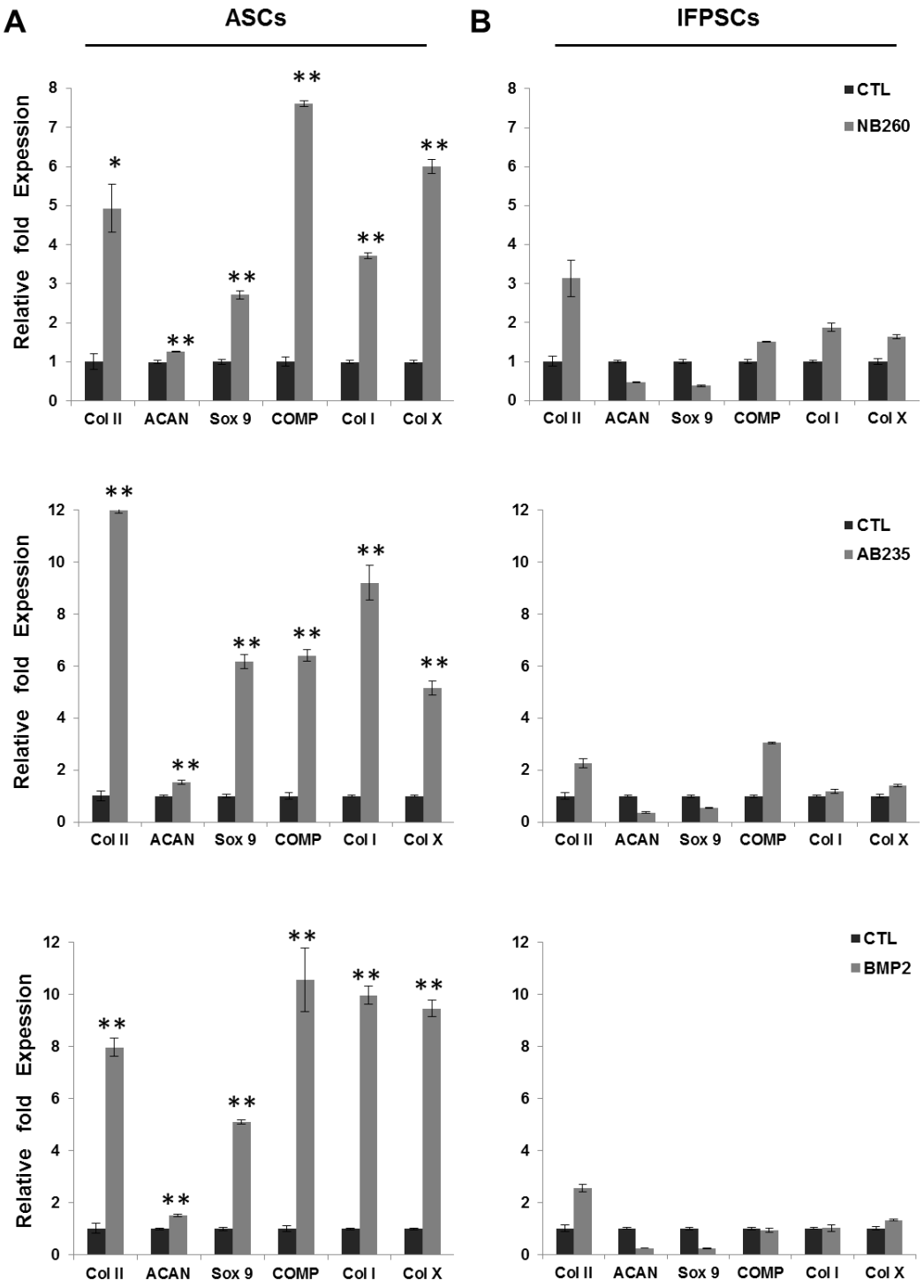

Fig. 10. Gene expression of chondrogenic markers. Real-time PCR analysis of selected chondrogenic markers after 6 weeks of NB260, AB235 and BMP2 treatment. All gene expressions were normalized with the values of untreated control pellets. Significant differences were found (* $p<0.05$ and ** $p<0.01$) in the expression of all chondrogenic markers when compared ASCs **(A)** with IFPSCs **(B)**.

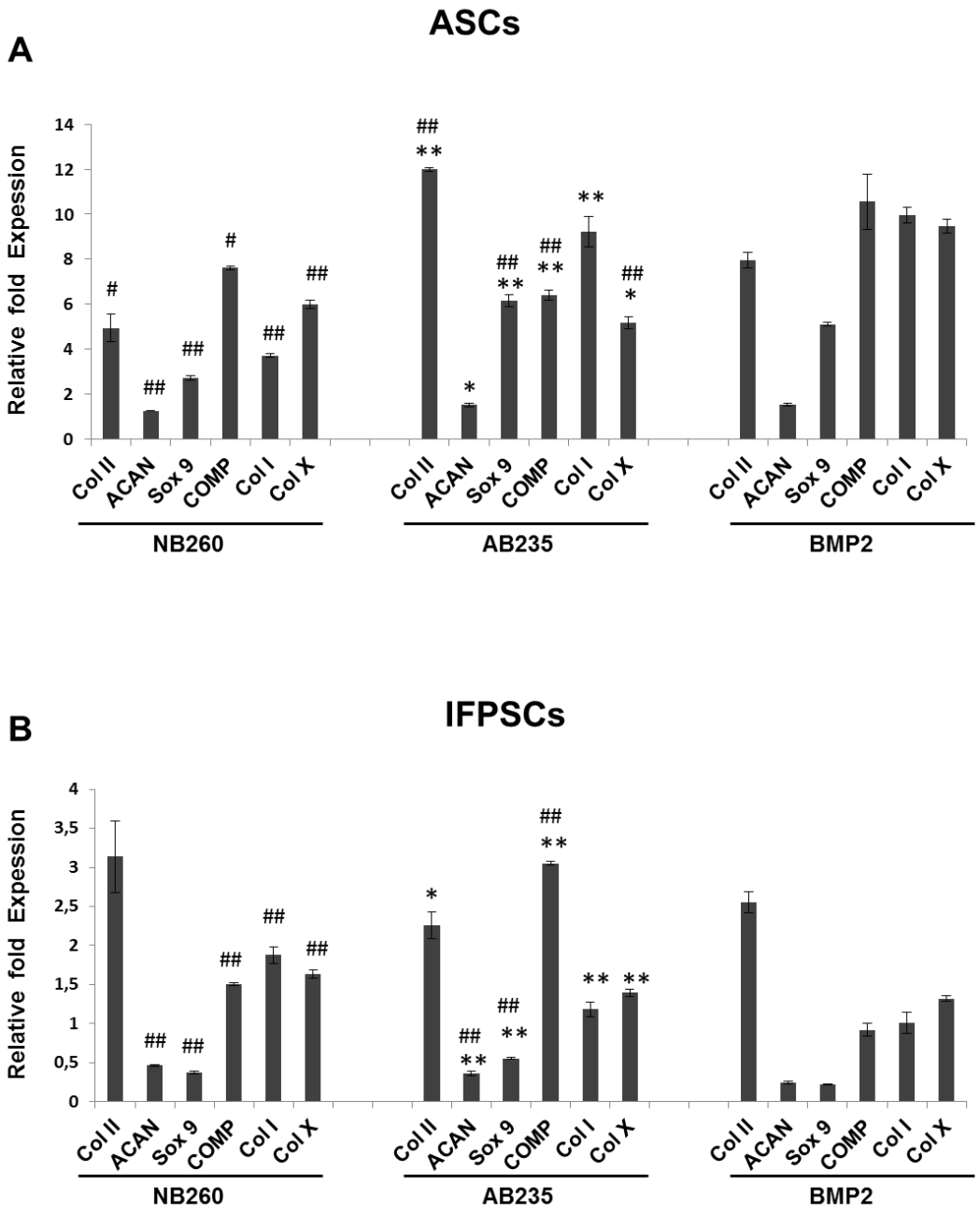

Fig. 11. Gene expression of chondrogenic markers. Real-time PCR analysis of selected chondrogenic markers after 6 weeks of NB260, AB235 and BMP2 treatment. All gene expressions were normalized with the values of untreated control pellets. Statistical significant differences were found (* $p<0.05$ and ** $p<0.01$) when compared gene expressions between NB260 and AB235 in ASCs **(A)** and IFPSCs **(B)**, and when compared NB260 and AB235 respect to BMP2 (#$p<0.05$ and ## $p<0.01$).

3. Adipose-derived stem cells treated with NB260 and AB235 promote cartilage integration upon transplantation in mice

We performed an *in vivo* assay to determine if the cartilage-like tissue growth after chimeric ligand induction could integrate into immunodeficient mice. Pellets were subcutaneously transplantated, and the stability of the new cartilage, the integration in the tissue and vascular invasion of the ectopic transplant were investigated.

Pellets obtained after 6 weeks of cultured in NB260, AB235 and BMP2-containing medium and untreated control medium, were implanted in subcutaneous tissue on the flanks of inmunodeficient mice. After 4 weeks, pellets were recovered for histological analysis.

ASCs pellets cultured with NB260 and AB235 chimeric ligands were recovery all, whereas in some cases, BMP2 treated pellets and untreated pellets were reabsorbed. On the other hand, IFPSCs pellets were recovered in less quantity that ASCs pellets, being NB260 pellets the most unstable because none could be harvested [Table. 3].

The fact that pellets were harvested demonstrated that the implant did not cause any rejection by the organism [Fig. 12A] Masson´s-Trichrome staining revealed a robust and dense ECM in treated pellets, in special AB235 pellets and without vessel passing through it, so the original cartilage phenotype remains [Fig. 12B]. Moreover, in AB235 treated pellets we could observe the formation of *novo* tissue around the pellet, demonstrating the integration the pellet in the organism.

Table 3 . Pellets recovered after 4 weeks of *in vivo* assay.

	CTL	**NB260**	**AB235**	**BMP2**
ASCs	2	3	3	1
IFPSCs	2	0	2	1

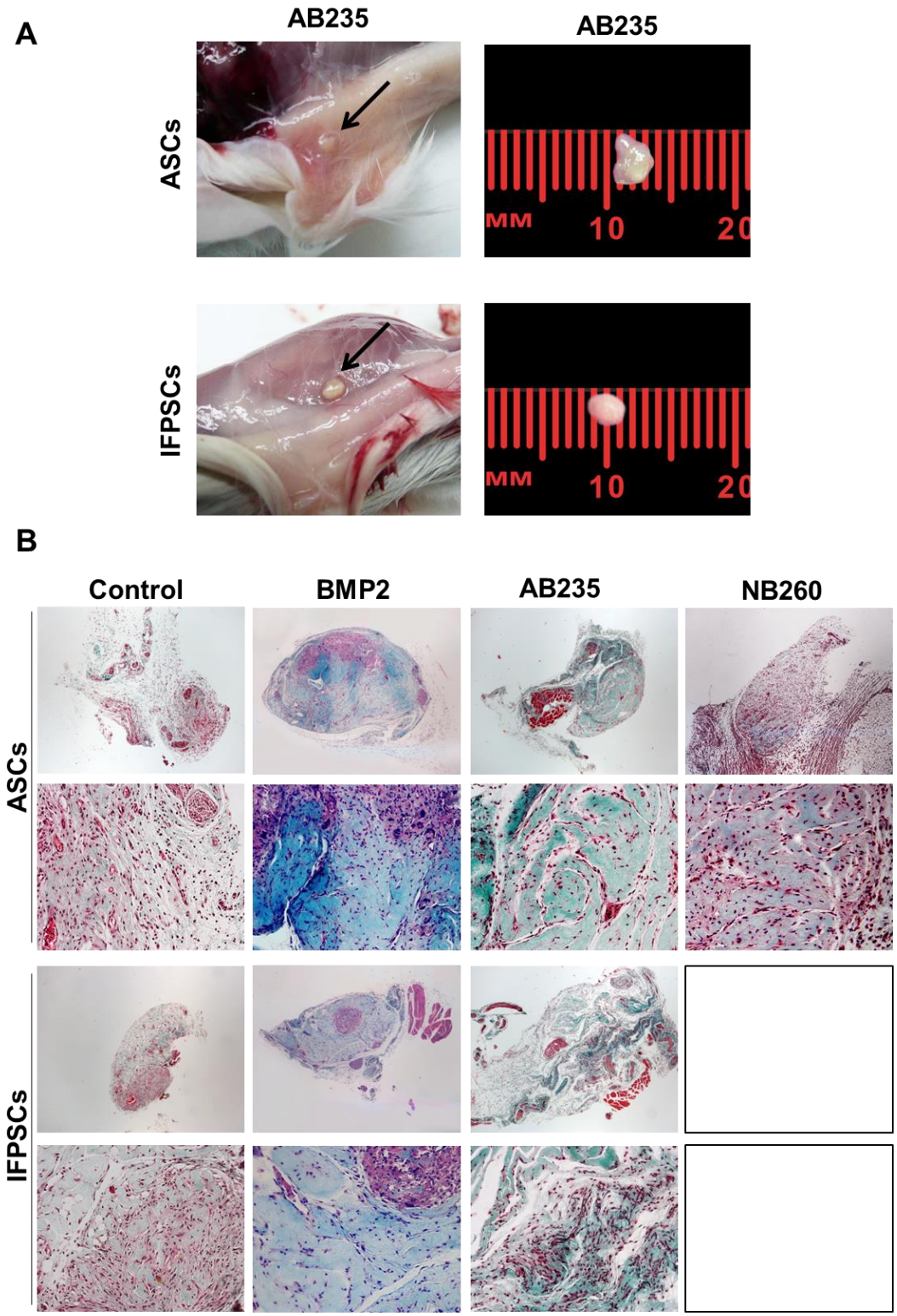

Fig. 12. In vivo integration of pellets cultured with chimerics ligands. **(A)** Integration of AB235 treated ASCs pellets and NB60 treated IFPs pellets with the surrounding tissue after 4 weeks of *in vivo* assay. **(B)** Pellets harvested from the mice and stained with Masson's Trichrome showed strong staining for collagens (green), distinctive of ECM of mature cartilage. Original magnification 10x and 20x.

Discussion

Hyaline articular cartilage has very low self-repair potential due to its intrinsic characteristics, therefore cell-based therapies directed to repair chondral lesions have emerged as a promising new approach. An example of cellular therapy that is currently used in clinic is autologous chondrocyte implantation (ACI), this technic consist in the implantation of *ex vivo* expanded chondrocytes, extracted from the own patient, into the injured area. However, the use of autologous chondrocytes for tissue regeneration is limited to treat small injuries since only a small amount of chondrocytes can be isolated from a patient biopsy. In order to increase cell number freshly isolated chondrocytes are *in vitro* expanded. Nevertheless, it has been proved that prolonged monolayer culture induces the loss of the chondrocyte phenotype (dedifferentiation) (Chung & Burdick 2008) (Bhosale & Richardson 2008). In this regard, adult mesenchymal stem cells (MSCs) that can be *in vitro* isolated, cultured and differentiated have emerged as an alternative and promising cell source for treat cartilage damage (Oldershaw 2012).

To induce chondrogenic differentiation of MSCs several growth factors and cytokines involved in the development, homeostasis and integrity of the cartilage have been tested. The key growths factors implicated in the process of chondrogenesis are: transforming growth factor-β (TGF-β), bone morphogenic proteins (BMPs), insulin-like growth factor (IGF), and fibroblast growth factor (FGF). Each of them is associated with specific signaling pathways, and induces the expression of specific genes involved in the chondrogenesis process (Yu et al. 2012) (Danišovič et al. 2012).

Activin, Nodal and BMP2 belong to the TGF-β superfamily, which are involved in numerous biological processes, including chondrogenic differentiation (W. Wang et al. 2014). Based in the knowledge of these specific chondrocyte pathways, rencently, it has been developed chimeras by mixing random Activin and BMP2 sequences (AB235: BAAAAA) and Nodal and BMP2 sequences (NB260: BNNNBN), using a swapping strategy of the segments called Random Assembly of Segmental Chimera and Heteromers (RASCH) (Allendorph et al. 2011) (Esquivies et al. 2014). TGFβ ligands are formed by six structurally distinct segments that conforms a conserve available crystal structure, so, for RASCH strategy each ligand was divided into six segments according

to its structure and re-emsambled to conform a new chimera, where the first segments is always from BMP2, because it was necessary for efficiently refolded (Kwiatkowski et al. 2014).

In the present study we compare the chondrogenic potential of adipose derived stem cells isolated from two different sources, i) from liposuctions (ASCs) and ii) from the infrapatellar fat pad of OA patients (IFPSCs). Both cell types were cultured under a chodrogenic induction protocol which consisted in a 3D pellet culture system and the exposure to NB260 and AB235 chimeric ligands. Moreover, we contrast the effect of each chimera in both adipose derive stem cells to establish the best combination of stem cells and chimeric ligand for cell therapy application.

First, isolated ASCs and IFPSCs were characterized and proved to fulfill the criteria of the ISCT (Dominici et al. 2006), thus both were plastic-adherent, did express mesenchymal markers and did not express hematopoietic or endothelial markers. In addition, the plasticity of the isolated cells was also confirmed.

Chondrogenic induction was performed under a 3D system model to facilitate the cell to cell contact necessary for the cellular condensation process required for cartilage formation (Barna & Niswander 2007), and to mimic the embryonic limb development with the aim to induce the formation of a cartilage-like tissue with the characteristic of native cartilage (Zhang et al. 2004).

The expression of specific proteins that conforms chondrogenesis was analyzed by histological and immunofluorescence studies. The enhanced expression of Col II, proteoglycans and Sox9 found in pellet sections that had been treated with AB235 or NB260 was similar to native cartilage tissue (Felimban et al. 2014). Moreover, differences in mRNA expression for chondrogenic and hypertrophic markers were also found when compared treated cells versus controls. Cartilage ECM is composed predominantly by collagens fibbers (Col II) and aggrecan (Dell'Accio et al. 2001) together with others important proteins such as COMP (Hedbom et al. 1992). In addition, the transcription factor Sox9 is also a key marker for early chondrogenesis.

Sox9 is expressed in chondroprogenitor cells and during mesenchymal condensations, being necessary for proliferation and differentiation of foetal chondrocytes in the growth plate. Furthermore, Sox9 promotes the expression of Col II, aggrecan and proteoglycans (Zhao et al. 1997) (Akiyama et al. 2002) (Lee et al. 2014) and acts as negative regulator of cartilage vascularization, endochondral ossification and osteogenic differentiation (Akiyama et al. 2002) (Hattori et al. 2010) (Liao et al. 2014).

In contrast, Col I and Col X are considered as negative markers in chondrogenesis. These proteins are related with endochondral ossification and hypertrophy. Although Col I is not a component of mature articular cartilage, it participates in regulating mesenchymal condensation and it is present in the first steps of the chondrogenic differentiation (Dessau et al. 1980) (Bobick et al. 2009). Moreover, Col I expression increase has been often closely related to chondrogenic differentiation in vitro (Tallheden et al. 2004). In addition, Mwale et al (2006) showed that an increment in mRNA of Col X in chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells, appeared even before that Col II, but they could not detect Col X protein, suggesting that mRNA Col X expression has no functional consequences (Mwale et al. 2006).

Here we show that NB260 and AB235 chimeric ligand induced, in both cell types tested, the upregulation of chondrogenic-markers genes. Moreover, hypertrophic markers showed lower expression in IFPSCs (Col I and Col X) and ASC (Col X) cultured with AB235 in comparison with NB260 treated pellets. We used standard BMP2 growth factor as control of chondrogenesis, and AB235 chimera improved the results obtained with BMP2, incrementing chondrogenic markers and maintaining or decreasing hypertrophic markers in both types of cells.

The higher chondrogenic potential of ASCs in relation with IFPSCs after treatment by chimeric ligands differs of the theory of micro-environmental niche, and the chondrogenic potential of IFPSCs associated with the proximity to the cartilage (Liu et al. 2014). However, results are not concluding because of determinations were done taken into account the basal gene expression for each adipose stem cells source. So,

basal levels were normalized with the values of untreated control pellets for ASCs and IFPSCs, respectively. In fact, it has been demonstrated that non-cartilaginous knee joint tissues such as IFP possess significant chondrogenic potentials and this may be associated with the proximity to the niche they reside (O'Sullivan et al. 2011). So, the higher chondrogenic potential of chimeras in ASCs than IFPSCs could be due to the previous high basal chondrocyte gene expression levels of IFPSCs due to the influence of intrarticular knee microenvironment.

TGFβ ligands are subdivided in two subfamilies: TGFβ and BMP. TGFβ subfamiliy ligands include TGFβ, Nodals, myostatin (GDF-8) and Mullerian inhibiting substance, and activate Smad2/3 transcription factors. On the other hand, BMP subfamily ligands consist in BMPs and growth and differentiation factors (GDFs) that activate Smad1/5/8 transcription factors (W. Wang et al. 2014). NB260 and AB235 chimeric ligand has the refolding efficiency of BMP2, and the signalling properties of Nodal and Activin repectively (Smad2/3), and it has not been reported about BMP2-like signalling through Smad1/5/8 (Allendorph et al. 2011) (Esquivies et al. 2014). BMPs ligands play an important role in mesenchymal condensation (Denker et al. 1999), and can induce differentiation into chondrocytes by activation of Sox9 gene expression (Yoon et al. 2005); however, distinct function of this pathway was observed after the onset of chondrogenesis, where Smad1/5/8 is associated with terminal differentiation, hypertrophy and calcification (Hellingman et al. 2011), whereas Smad2/3 participate in Col II deposition, retrains chondrocytes terminal differentiation and is considerate as protective for articular cartilage (van der Kraan 2014) (Madej et al. 2014) (Ferguson et al. 2000).

The higher chondrogenic potential of AB235 may be due to its intrinsic characteristics. Nodal in NB260 chimera including dependence on the co-receptor Cripto to initiate Smad pathways (Esquivies et al. 2014), while Activin A in AB235 is independent of the co-receptor Cripto (Peran et al. 2013), which has different effect in Activin A, playing as an inhibitor (Kelber et al. 2008). Furthermore, Activin A has been shown to be an inhibitor of catabolic process that leads to the degradation of the ECM (Chang et al. 2007) (Alexander et al. 2007). Another difference between both chimeras is that

AB235 presents a mutation created to make AB235 Noggin sensitive (Peran et al. 2013). Noggin protein is an inhibitor of BMPs ligands, expressed during limb development (Capdevila & Johnson 1998) (Brunet et al. 1998) (Xie et al. 2015), that abolishes BMPs signalling by binding directly to BMPs (Groppe et al. 2002). A good regulation of BMPs is necessary because when it is found at high concentrations it induces osteogenic differentiation (De Luca F et al. 2001) (Valcourt et al. 2002). These facts, could explain the better chondrogenic potential of AB235 in comparison with BMP2.

Finally, we proved the integration capacity of the induced cartilage pellets into an *in vivo* model. For this purpose, treated and untreated control pellets were transplanted into the flanks of immunodeficient mice and recovered four weeks later. ASCs pellets cultured with AB235 chimera presented a complex ECM similar to native cartilage and *in vivo* adequate integration, even improving the chondrogenic potential of BMP2 used in this experiments as positive control treatment (Sekiya et al. 2005) (Wei et al. 2006). By contrast, not all IFPSCs could be recovered and IFPSCs pellets were smaller and less compact than ASCs pellets.

In conclusion, we have demonstrated that human adipose derived stem cells isolated from liposuctions (ASC) presents higher chondrogenic potential that those isolated from the infrapatellar fat pad (IFPSCs) of OA patients. Stimulating MSCs with conventional growth factors and cytokines are expensive, and usually requires high concentration and repeated treatments that increment the costs. Our results present an efficient and reproducible protocol of chondrogenic differentiation based on the use of AB235 chimeric ligand, which could have a therapeutic potential in patients with OA.

CHAPTER II

Chondrogenic redifferentiation of dedifferentiated chondrocytes

Results

1. Chondrocyte dedifferentiation upon monolayer culture

In order to establish the potential of dedifferentiated chondrocytes to revert their phenotype to a chondrocyte mature state, chondrocytes were isolated from articular cartilage samples obtained from patients with knee OA during joint replacement surgery. To minimize the effects of OA, only cartilage that macroscopically looked relatively normal was used for this study

Freshly isolated chondrocytes were grown in monolayer culture up to passage 6 (P6) to ensure a complete dedifferentiation. Dedifferentiation was evident as soon as P3, when the proliferation of the cells increased and some chondrocytes started to change morphology and adopt a spindle-like fibroblastic shape. After 4 weeks all cells had increased in size and adopted a fibroblast-like appearance [Fig. 13A], while control chondrocytes cultured for only 7 days retained a typical polygonal and star-shaped morphology. Toluidine Blue staining, which reflects synthesis of GAGs, was clearly decreased in cell monolayers at P6 relative to control chondrocytes [Fig. 13A]. Further, chondrocytes cultured for 4 weeks showed decreased Col II expression (red staining) and increased Col I expression (green staining) while control chondrocytes showed the opposite [Fig. 13A].

Finally, we evaluated the expression of selected chondrogenic markers by real-time PCR [Fig. 13B] and found that chondrocytes at P6 had lost the expression of typical chondogenic markers, decrease expression of Col II ($p < 0.01$) with concomitant increase in expression of Col I and Col X ($p < 0.01$) when compared with control cells [Fig. 13B]. A slight increase of Sox9 expression ($p < 0.01$) was also detected specifically in long-term cultured chondrocytes. Furthermore, a significant decrease of the Col II/Col I ratio ($p<0.01$) was observed in chondrocytes at P6 when compared to the ratio in the control cells [Fig. 13B]. Together, these results indicate chondrocytes cultured in monolayer for four weeks adopt a dedifferentiated, fibroblast-like phenotype.

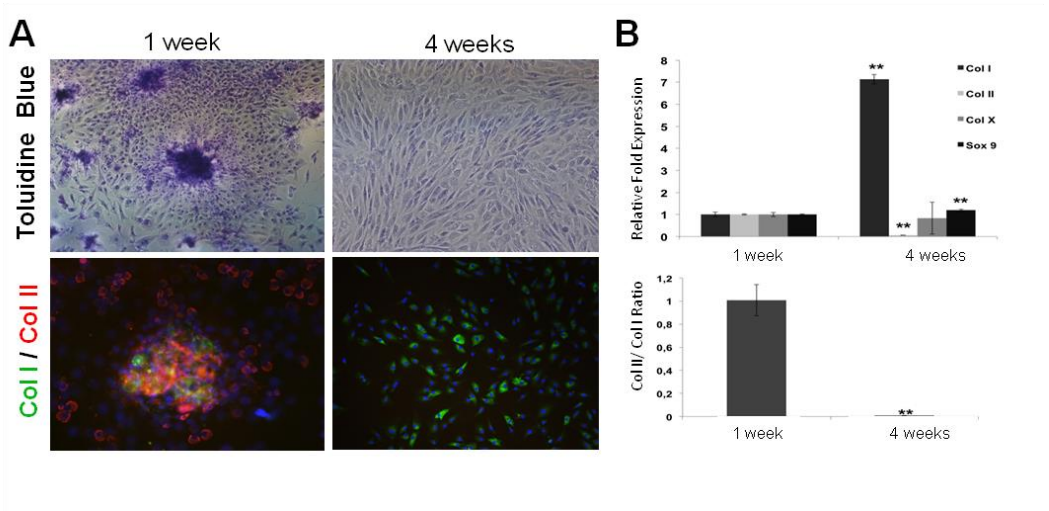

Fig. 13. Dedifferentiation of chondrocyes grown in monolayer culture. **(A)** Chondrocytes cultured for 1 week or 4 weeks were stained with Toluidine Blue and inmunolabelled for Col I (green) and Col II (red). **(B)** Real-time PCR analysis of selected chondrogenic markers after 4 weeks of monolayer cell culture. The bottom graphic shows the ratio of Col II versus Col I expression during the process of differentiation. **Statistical significance indicated ($p<0.01$).

2. AB235 promotes redifferentiation of dedifferentiated chondrocytes

Previously, we had established the chondrogenic potential of Activin/BMP2 chimeric ligand (AB235) for to induce differentiation towards chondrocyte lineage in adipose-derived stem cells. In this study, we tested if the chimeric AB235 ligand could induce redifferentiation of chondrocytes that have lost their differentiated phenotype following extended monolayer culture.

Dedifferentiated cells were cultured for 4 weeks under pellet-forming conditions as described previously (Peran et al. 2013). Briefly, cells were grown in monolayer for two weeks, and later, cells treated with AB235 and untreated control cells were manually separated using a sterile scraper, transferred to conic tubes and cultured under pellet system conditions for four weeks. After a total of 6 weeks, cell pellets cultured in media containing AB235 or without treatment were processed for analyzed using histological and immunofluorescence probes. BMP2 was used as positive control.

Pellets cultured in AB235-containing medium showed a noticeable increase in size relative to untreated controls. In addition, the AB235-induced pellets had a consistency and appearance more similar to native cartilage tissue [Fig. 14A]. We compared the internal structure of the AB235-treated and non-treated pellets and found that the ECM of treated pellets was also more similar to that of native cartilage tissue [Fig.14B]. Pellet sections stained for Hematoxilyn-Eosin showed that AB235 induced the formation of a complex cellular organization with cells embedded in lacunae surrounded by ECM (staining in pale pink), again resembling native cartilage tissue, while ECM was not visible when cells were cultured under control conditions. Masson´s-Trichrome staining revealed collagen-specific staining (green) in the ECM of AB235-treated pellet sections that was similar to the staining of the native tissue but not visible in control pellets. Cartilage specific proteoglycans were also clearly more apparent in AB235-treated pellets than in the control as assessed by Alcian Blue and Toluidine Blue assays pellets.

Immunofluorescence analysis displayed significantly higher levels of protein expression for cartilage ECM markers Col II, and aggrecan, as well as, transcription factor Sox 9, the key marker for chondrogenesis, in AB235-induced pellets than in the control pellets that showed weak and diffuse staining for these markers of chondrocyte differentiation [Fig. 14C]. The patterned arrangement of Col II and aggrecan fibres is striking and in stark contrast with the homogeneous distribution of this protein in control pellet sections.

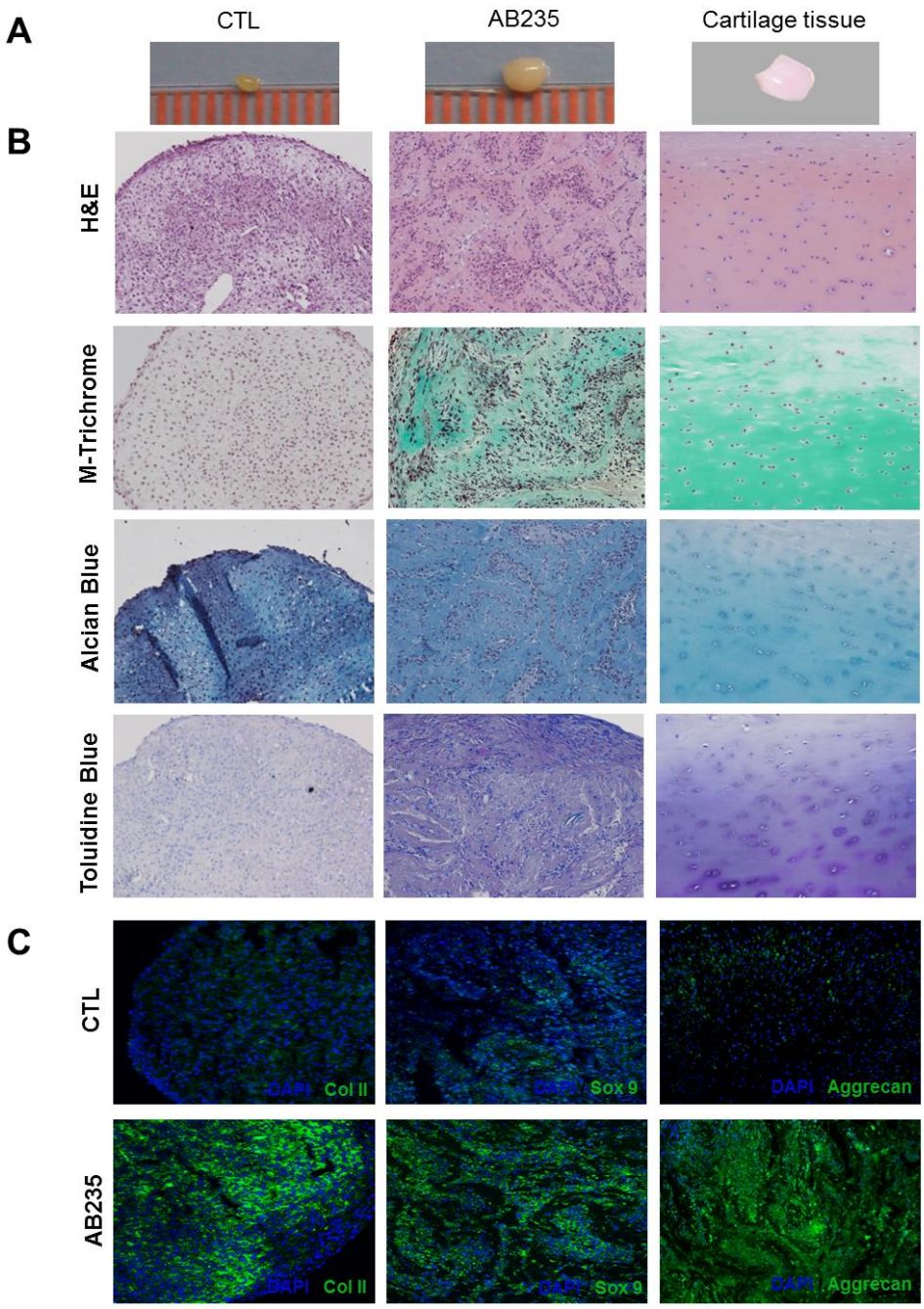

Fig. 14. AB235 induces chondrocyte redifferentation *in vitro*. (A) Representative images of dedifferentiated chondrocytes cultured in a pellet system for 6 weeks in the absence of treatment (CTL) or treated with 10 ng/ml of the chimeric ligand (AB235) are compared with an image of native cartilage tissue. **(B)** Histological staining of sections of the pellets from (A) shows the acquisition of a cartilage like matrix resulting from AB235 treatment. **(C)** Merged images of pellets sections inmunostained with Col II, Sox9 and Aggrecan antibodies (green channel) and cell nuclei labelled with DAPI (blue channel) demonstrate that AB235 treatment increases of chondrogenic marker expression. Original magnification: 20x for all panels.

Further, quantitative image analysis was performed using ImageJ. For histological and immufluorescence images, the staining of AB235-treated pellets was significantly higher ($p < 0.01$) in all cases when compared with control pellets [Fig. 15].

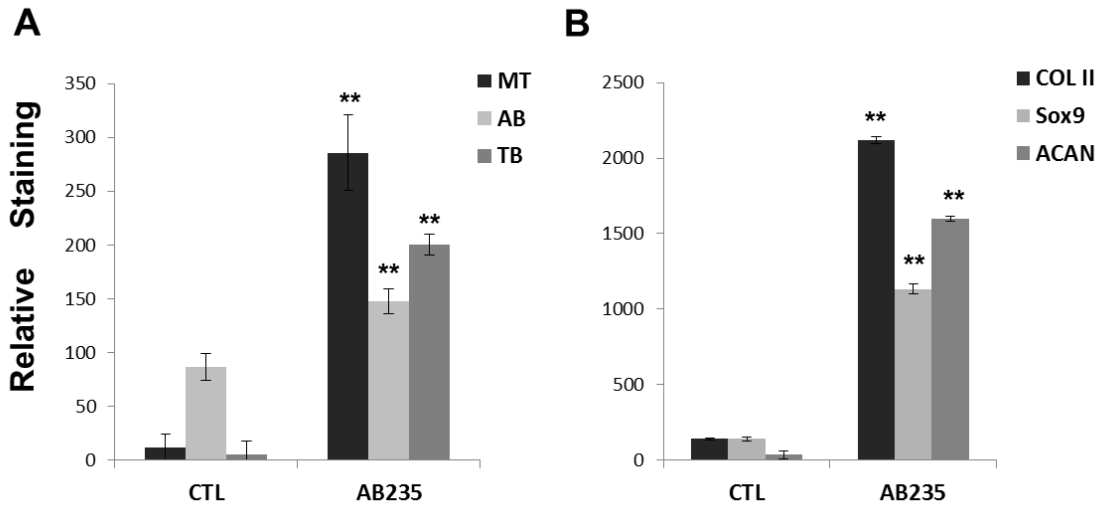

Fig. 15. Quantitative image analysis. (A) Graphical representation of the quantification of hitological and **(B)** immunofluorescence staining of control and AB235-treated pellet sections. MT: Masson-Trichrome; AB: Alcian Blue; TB: Toluidine Blue; ACAN: Aggrecan. **Statistical significance indicated ($p<0.01$).

Together, these data indicate that AB235 promotes re-establishment of the chondrocytic phenotype in cells that lose this phenotype following extended culture periods *in vitro*.

3. Chondrocytes *in vitro* redifferentiated by AB235 promote cartilage integration upon transplantation in mice

We tested whether chondrocytes redifferentiated as a pellet *in vitro* are capable of maintaining their 3D structure after being transplanted into mice. Pellets obtained after 6 weeks of culture in the presence or absence of AB235 were transplanted into subcutaneous tissue on the flanks of inmunodeficient mice and then harvested 4 weeks later for histological and immunofluorescence analysis.

We find that control pellets are completely absorbed by the surrounding mouse tissue and could not be recovered for histological and immunofluorescence analysis. By contrast, AB235-treated pellets displayed a dramatic increase in size over the 4 week period demonstrating that the graft was well tolerated by the organism [Fig. 16A].

Histological analysis reveals that the ECM synthesized by redifferentiated chondrocytes is cartilage-specific pericellular matrix consisting primarily of vertically-oriented collagen fibres and proteoglycans [Fig. 16B]. Integration of the AB235-treated pellet into the surrounding mouse tissue is demonstrated by the formation of *novo* tissue around the pellet that can be seen in the Hematoxylin-Eosin stained section as shown in Figure 16B. Finally, our immunofluorescence assay for Col I and Col X showed that AB235 treatment does not induce fibrotic or hypertrophic cartilage formation [Fig. 16 C-D]. On the other hand, Col II and Sox9 markers were highly expressed with an arranged Col II distribution typical of a structured ECM and with Sox9 localized in both the nucleus and cytoplasm [Fig. 16 E-F]. Finally, our results showed that Col X was almost undetectable in AB235-treated cells.

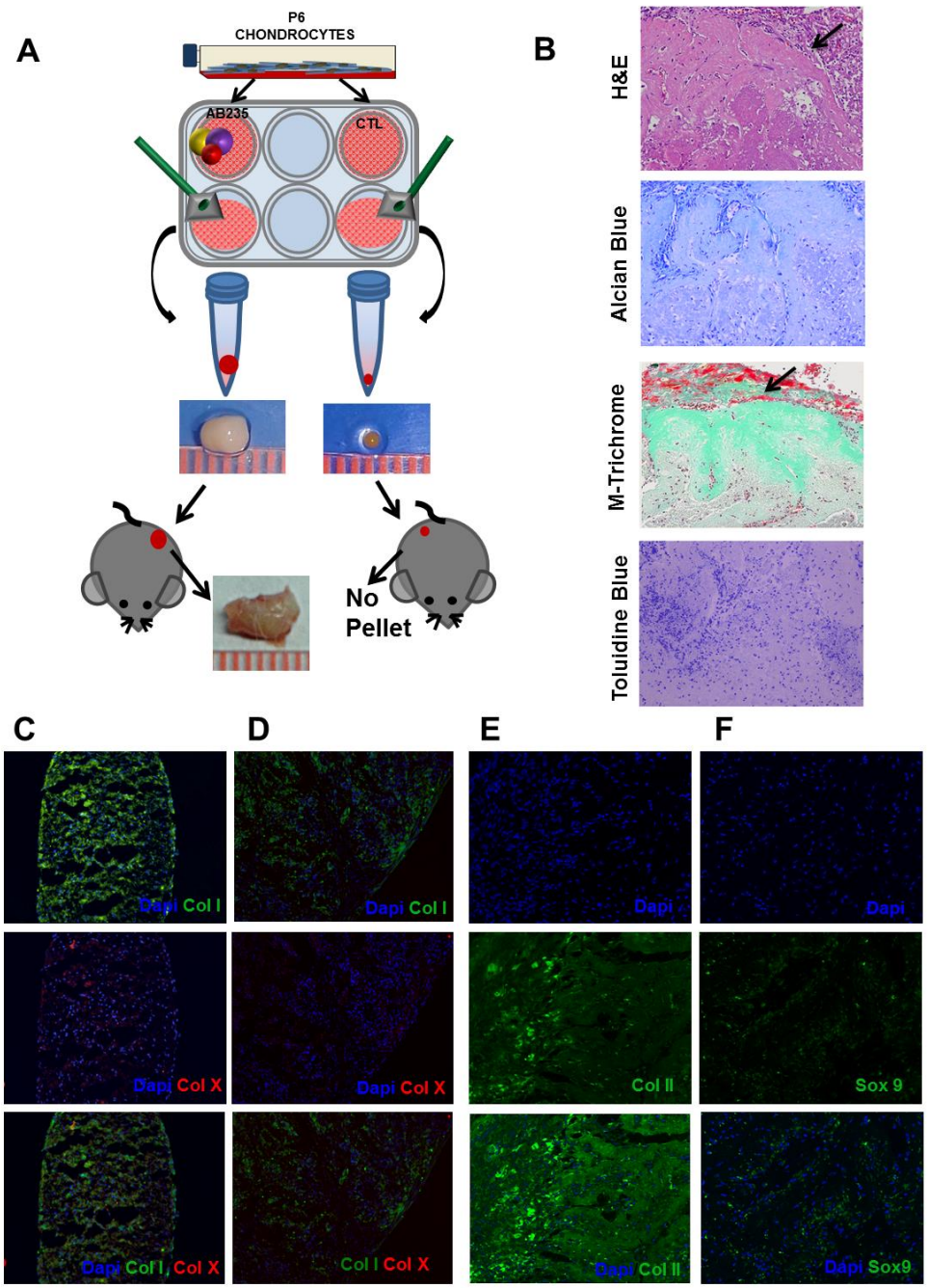

Fig. 16. AB235 induces chondrocyte redifferentiation *in vivo*. (A) Schematic representation of the experimental design showing images of representative pellets before and after implantation into mice. Integration of AB235 treated pellets with the surrounding tissue. **(B)** Sections of AB235-treated pellets harvested from mice and stained for H&E, Masson's Trichrome, Alcian Blue and Toluidine Blue show a robust staining for mature, cartilage-like ECM. Black arrows indicate the edge of the pellet in H&E and Masson's Trichrome stained sections while the edge of the pellet is not visible in alcian blue and touludin blue stained

sections. **(C-F)** Representative images of immunofluorescence analysis of cartilage markers. Stained sections of fibrotic marker type I collagen (Col I) and hypertrophic marker type X collagen (Col X) in both control pellet grown *in vitro* **(C)** and AB235-induced pellet harvested from mice **(D)**. Expression of the chondrogenic markers Col II and Sox9 in AB235 induced pellet sections after the in vivo assay **(E and F)**. Original magnification 10x for C and D; 20x for E and F.

Discussion

Autologous chondrocytes are suitable for cell therapy strategies directed to repair cartilage tissue degeneration or damage. However, these strategies are hampered by the fact that chondrocytes undergo dedifferentiation when they are grown in monolayer culture for prolonged periods.

Chondrocyte dedifferentiation has been described to occur as soon as 4-10 days after cells are plated in a monolayer (Barlic et al. 2008). We ensured full dedifferentiation toward a fibroblastic phenotype by growing chondrocytes in monolayer culture over a period of 4 weeks. Morphological, histological and immunological analysis together with real-time PCR measurement of Col I and Col II gene expression confirmed the complete dedifferentiation of chondrocytes over this 4 week period in a manner that is in agreement with prior findings (Martin et al. 2001).

We have previously reported the creation of chimeric ligands that combine BMP2 and Activin A sequences (Allendorph et al. 2011). We subsequently confirmed the chondrogenic potential of Activin and Nodal-like chimeras including AB235 in directing chondrogenic differentiation of adipose derived stem cells (Peran et al. 2013) (Esquivies et al. 2014) . In the present study, we specifically hypothesized that AB235 reverts dedifferentiated chondrocytes back to their previous, fully differentiated chondrocytic state. We tested this hypothesis by culturing dedifferentiated chondrocytes under 3D conditions. It is known that cell-to-cell contact promotes chondrogenic differentiation and 3D culturing has been used before to induce redifferentiation of monolayer-expanded autologous chondrocytes (Caron et al. 2012). However, our results showed that 3D culturing is not sufficient *per se* to redifferentiate chondrocytes as proven by the small size and lack of proper tissue organization found in the control pellets.

On the other hand, pellets cultured with AB235 produced a cartilaginous matrix comparable to the ECM found in native cartilage tissue. These findings suggest that the AB235 ligand interacts with Activin/BMP receptors in a way that promotes the reversion toward a chondrocytic phenotype in a manner concordant with what previous studies using BMP2 have shown (Salentey et al. 2009) (Cha et al. 2013). In

fact, the enhanced chondrogenic differentiation of human adipose derived stem cells (hASCs) treated with AB235 relative to that of cells treated with BMP2 suggests that this chimeric ligand signals more efficiently than BMP2 through type I and type II receptors that mediate cartilage maturation (Peran et al. 2013).

Furthermore, we demonstrate that AB235-induced cartilage integrates into the subcutaneous tissue upon transplantation into the flanks of immune compromised mice and that the structural cartilage-like complexity of the ECM in AB235-treated pellets strongly resembles native cartilage. By contrast, non-treated control pellets were absorbed by the surrounding tissue and could not be recovered for examination, in agreement with others studies that have showed that dedifferentiated chondrocytes failed to form cartilage tissue *in vivo* (Kreuz et al. 2013) (Dell'Accio et al. 2001). Interestingly, Activin A has been shown to be an inhibitor of matrix metalloproteinase 3 and to block the degradation of the ECM by this enzyme (Chang et al. 2007) raising the possibility that AB235, which utilizes the activin pathway, may exert similar effects.

From the present study we can conclude that the combination of sequences of BMP2 and Activin-A present in AB235 result in a ligand that increases the expression of chondrogenic markers of dedifferentiated chondrocytes. Others studies have shown that BMP2 upregulates chondrogenic gene expression of human articular chondrocytes expanded *in vitro* (Murphy et al. 2015) and, therefore, comparing the chondrogenic potential of BMP2 versus AB235 would be of great interest for future studies.

In conclusion, we describe an effective protocol for redifferentiation of autologous chondrocytes obtained from OA patients and the formation of a cartilage-like ECM that can integrate into the surrounding tissue *in vivo*. Future work will include assessment of the tumour-forming potential of AB235-treated cells in order to determine if the procedure can be translated to the clinic. Since the success of cell therapies for cartilage injury depends on the quality and quantity of the implanted cells, our protocol may have significant potential for clinical applications.

CHAPTER III

Synthetic polymers for cartilage tissue engineering

Results

1. Polymer microarray analysis

The objective of this study was to evaluate a large number of different synthetic polymers to select those that favour the adhesion and proliferation of chondrocytes, and the maintenance in culture of a differentiated phenotype for prolonged periods of time. For this proposal, we have used the polymer microarray approach developed by Professor Mark Bradley's group. Polymer microarrays were processed as previously reported by Pernagallo et al (2011) (Pernagallo & Diaz-Mochon 2011) to allow the screening of 380 different novel polymers, composed by polyurethanes (PU) and polyacrylates (PA) distributed as spots in a glass slide.

1.1. Analysis of cell attachment, viability and competitive affinity.

To determine if the polymer composition could influence specific cell attachment we tested different cell types to select the best polymer in each case. Fresh isolated chondrocytes, ASCs and IFPSCs, were seeded onto polymer microarrays to screen those that promoted cell adhesion and support cell viability. In addition, we tested if the *in vitro* induction of chondrogenic differentiation of ASCs and IFPSCs could change polymer affinity.

Cells were cultured onto the polymer microarray for 72 hours and them fixed and stained with DAPI to quantify cell attachment by counting the number of cells in each spot. Furthermore, the vital dye CTG was used to test polymer biocompatibility [Fig. 18].

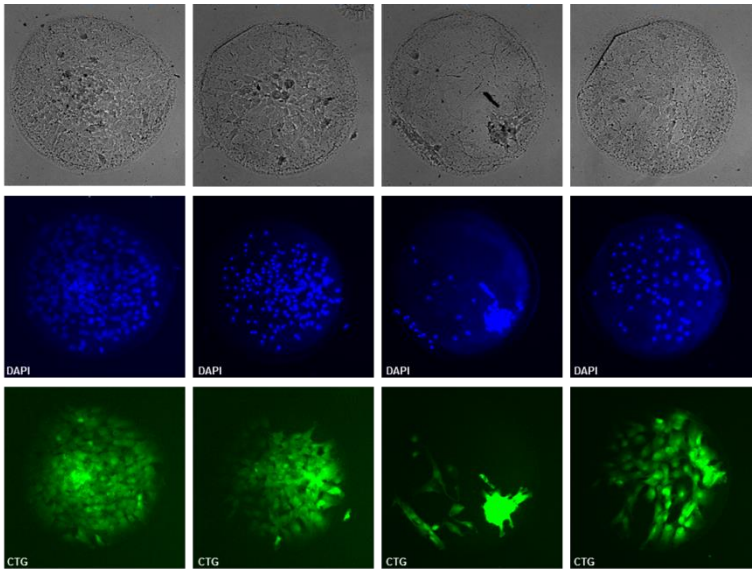

Fig. 18. Analysis of cell attachment, viability and competitive affinity in polymer microarray. Phase-contrast light microscopy of cultured chondrocytes during 72 hours in polymer microarrays. Example of spots 109-112 corresponding to PA204 polymer. Spots staining with DAPI (blue) and CTG (green).

From the analysis of cell adhesion and cell viability it was concluded that PA polymers supported chondrocyte adhesion significantly more than polymers composed by PU. The ten best polymers were chosen based on the number of cells attached per spot and only one PU polymer was included in the shortlist. Table 4 summarizes the average of cells per spot in each of the selected top 10 polymers. It can be appreciate that PA167 seemed to be the best substrate, providing an average of 136 chondrocytes per spot, followed closely by PU153 with 131 chondrocytes, and others PA polymers such as: PA204, PA234, PA391, PA202, PA410, PA309, PA520 and PA460.

Although IFPSCs and ASCs showed adherence to the top 10polymers, the number of cells per spot was lower for all polymers when compared with the number of chondrocytes [Table 4]. Only PA202 showed a slight increment of IFPSCs number when compared with the chondrocytes. In one polymer, PA204, the differentiation of ASCs toward a chondrogenic phenotype by the addition of AB235 seemed to increased cell attachment.

Table 4 -. Best 10 selected polymers from the polymer microarray.

	Number of cells					% of cells				
Polymer	**IFPSCs – AB235**	**IFPSCs**	**ASCs**	**ASCs – AB235**	**Chondrocytes**	**IFPSCs – AB235**	**IFPSCs**	**ASCs**	**ASCs – AB235**	**Chondrocytes**
PA167	25	30	1	1	136	64	34	2	1	100
PU153	1	1	20	9	131	3	1	34	13	96
PA204	20	41	59	72	123	52	46	100	100	91
PA234	25	56	23	34	108	65	63	39	47	79
PA391	10	23	39	39	95	26	26	66	54	70
PA202	1	89	47	22	79	3	100	80	31	58
PA410	1	1	1	1	78	3	1	2	1	58
PA309	1	18	36	23	47	3	20	60	31	35
PA520	1	1	4	21	43	3	1	7	29	32
PA460	26	6	8	2	39	68	7	13	3	29

1.2. Analysis of cell proliferation and chondrogenic potential.

The top 10 polymers selected were subjected to further assays to select the best polymer composition to support chondrocyte phenotype and proliferation. For this purpose, 12 well plates were coated with the top 10 polymer solutions (dissolve at 2.0% w/v in acetic acid) and chondrocytes were cultured during 10 days. At day 2, 4, 7 and 10 phase-contrast light microscopy images were taken to evaluate cell proliferation in each polymer [Fig. 18-23]. Moreover, chondrogenic and osteogenic potential of polymers was analyzed at 10 days by Toluidine Blue and Alizared Red staining, respectively. Uncoated wells were used as control.

Analysis revealed that PA167 did not support any type of cell adhesion, and in PA410 and PA460 chondrocytes were found only in the edge of the wells, for these reasons these 3 polymers were discarded. The rest of 7 polymers, PU153, PA294, PA234,

PA391, PA202, PA309 and PA520, presented similar cell adhesion and cell proliferation between them [Fig. 19]. Furthermore, the presence of GAG, a cartilage-specific ECM components, was confirmed by Toluidine Blue staining, which reveals that the attached chondrocytes maintained their differentiated phenotype.

In addition, we found that PU153, PA204, PA234, PA391, PA202, PA309 and PA520 polymers support IFPSCs and ASCs attachment and proliferation and a weak increase in GAGs deposition [Fig. 19 and Fig. 21]. Interestingly, IFSPCs and ASCs that had been previously differentiated toward a chondrocyte-like phenotype, with the chimeric ligand AB235, showed a proliferation pattern similar to chondrocytes and´, also presented an ECM rich in GAG as revealed the Toluidine Blue staining [Fig. 22 and Fig. 23].

Finally, Alizarin Red staining was performed to check the capacity to induce osteogenic differentiation of the top 10 polymers. Just PA410 polymer showed a light positive staining for typical calcium deposits [Fig. 18-23].

Once we selected 7 top polymers on base to cell adhesion, biocompatibility and chondrogenic ECM maintenance, we further shorter up the list of polymers taking into account other considerations such as availability, the easiness to produce the polymer in large amounts and their dissolve properties. Finally, two polymers PA204 and PA391 were proved to fulfill the characteristics above cited and were used for the subsequent studies.

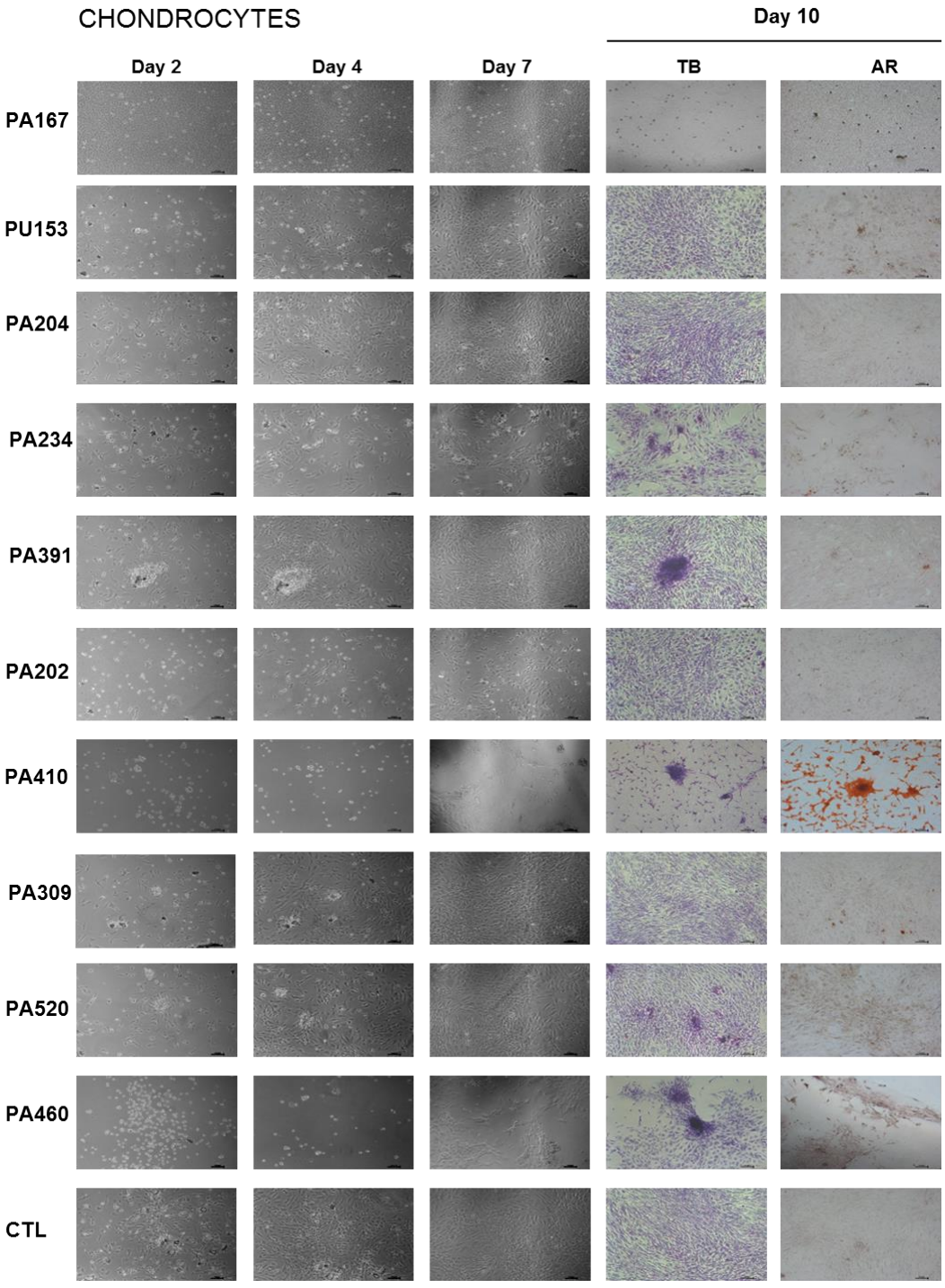

Fig. 19. Analysis of cell proliferation and chondrogenic potential. Proliferation assay in twelve well plates cover with the best 10 polymers. At 10 days Toluidine Blue (TB) and Alizarin Red (AR) were done to check chondrocyte or osteoblast phenotype, respectively.

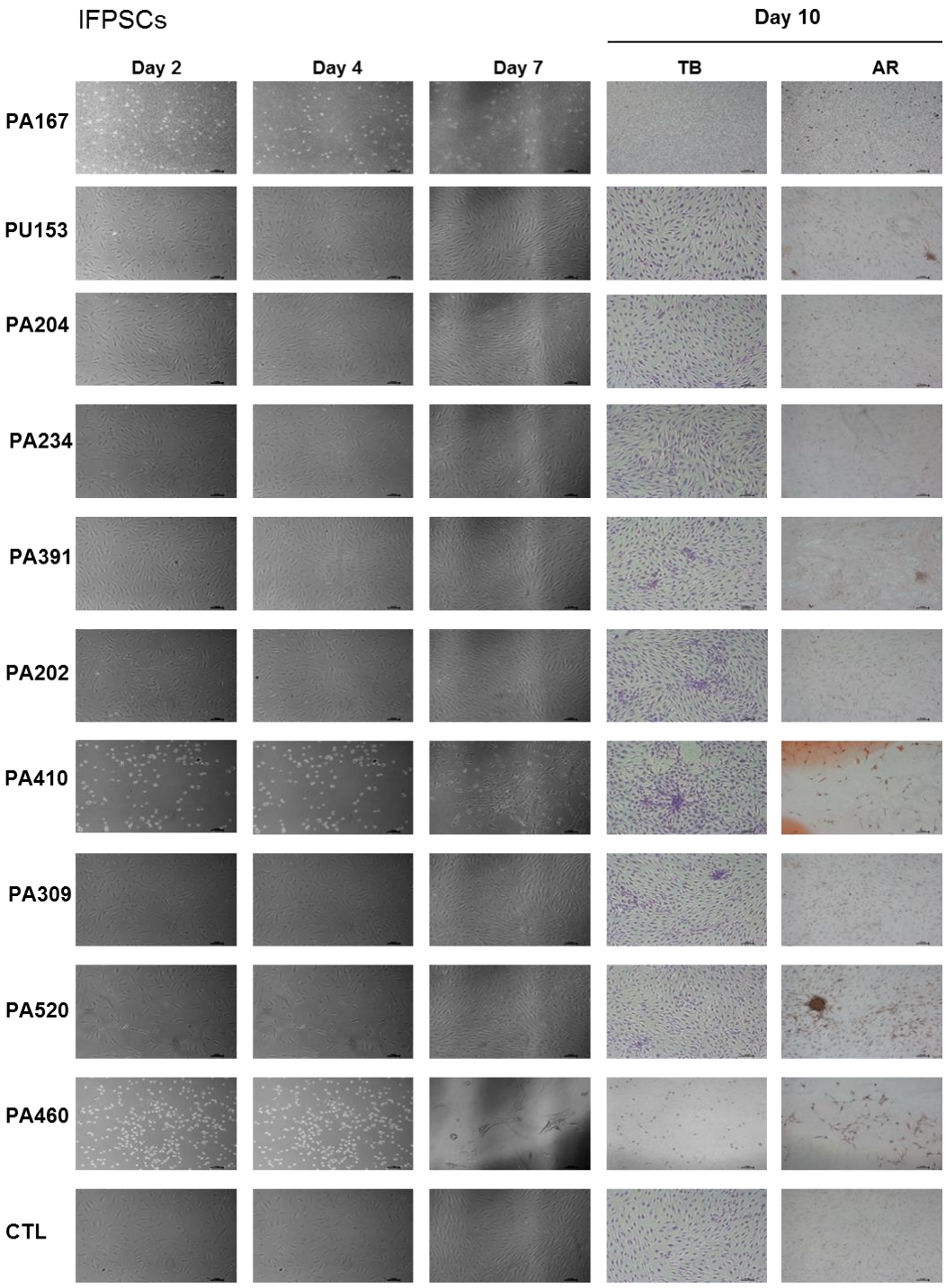

Fig. 20. Analysis of cell proliferation and chondrogenic potential of IFPSCs. Proliferation assay in twelve well plates cover with the best 10 polymers. At 10 days Toluidine Blue (TB) and Alizarin Red (AR) were done to check chondrocyte or osteoblast phenotype, respectively.

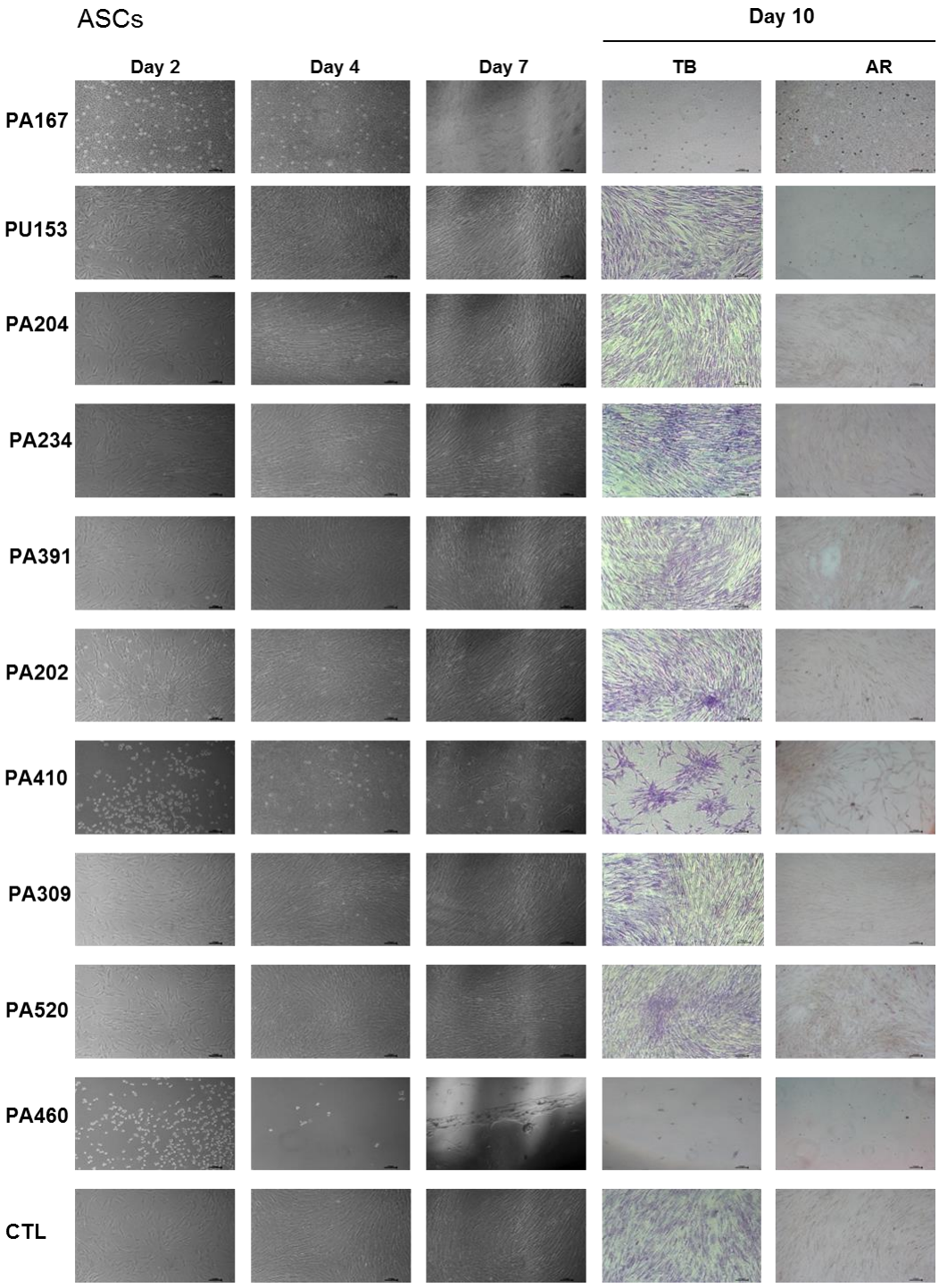

Fig. 21. Analysis of cell proliferation and chondrogenic potential of ASCs. Proliferation assay in twelve well plates cover with the best 10 polymers. At 10 days Toluidine Blue (TB) and Alizarin Red (AR) were done to check chondrocyte or osteoblast phenotype respectively.

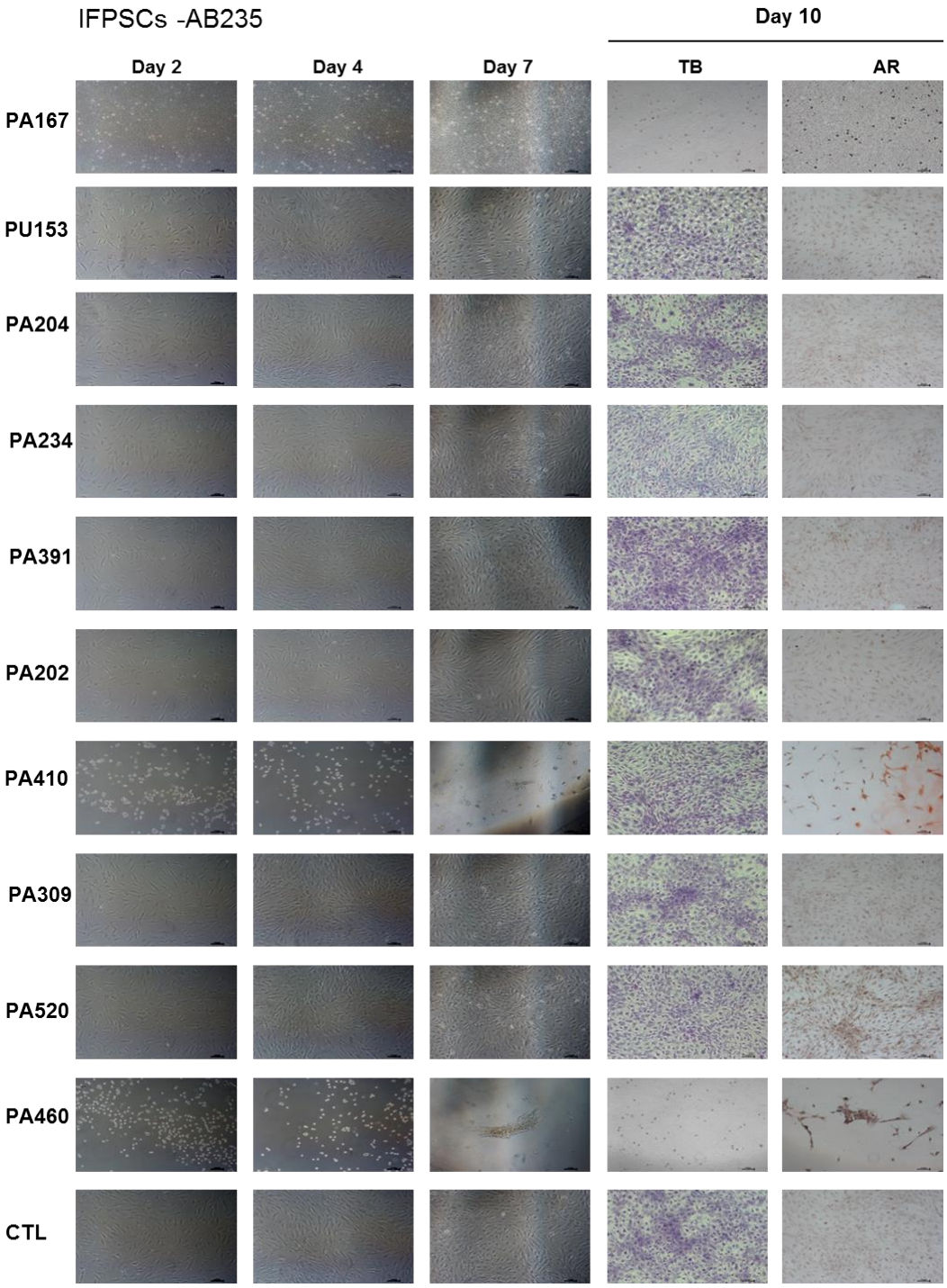

Fig. 22. Analysis of cell proliferation and chondrogenic induction of IFPSCs cultured with AB235. Proliferation assay in twelve well plates cover with the best 10 polymers. At 10 days Toluidine Blue (TB) and Alizarin Red (AR) were done to check chondrocyte or osteoblast phenotype respectively.

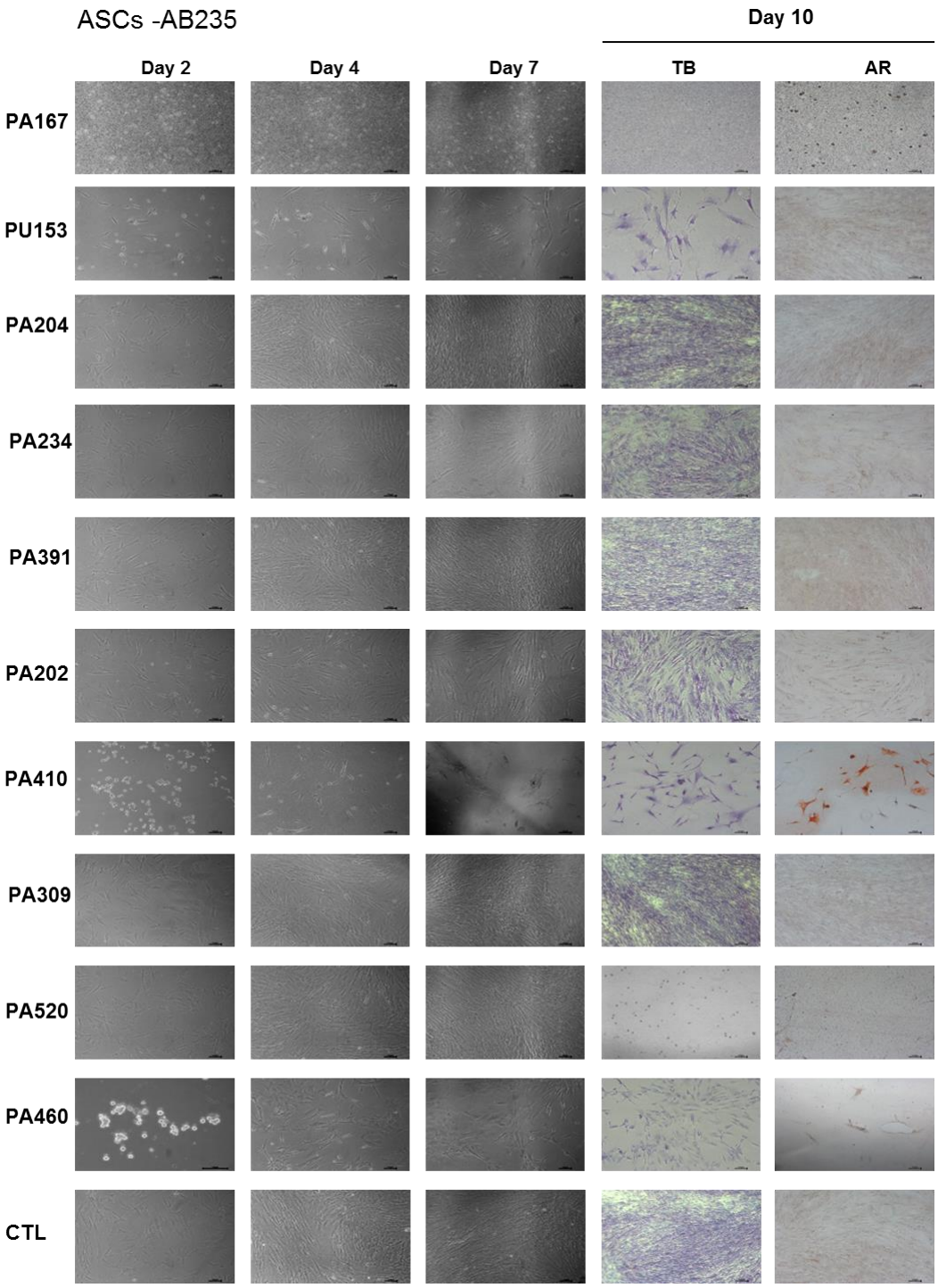

Fig. 23. Analysis of cell proliferation and chondrogenic induction of ASCs cultured with AB235. Proliferation assay in twelve well plates cover with the best 10 polymers. At 10 days Toluidine Blue (TB) and Alizarin Red (AR) were done to check chondrocyte or osteoblast phenotype respectively.

2. Hydrogels

To mimic the natural 3D microenvironment of the cellular niche we prepared hydrogel scaffolds using the 2 polymers selected. Hydrogels were made by chemical crosslinked using radical polymerization. TEMED, PA204 and PA391 polymers were dissolved in NMP, together with one of the three crosslinkers tested: PEGDA, PEG and PETA (see table 3). The hydrogel polymer synthesis was achieved by adding APS as oxidation-reduction initiator that together with TEMED (co-initiator) allowed the copolymerization of the monomers and the crosslinkers. Hydrogels were made to achieve a cylindrical shape with an approximately height and diameter of 4 and 8 mm, respectively.

First, scaffold hydrogels biocompatibility was assessed. Chondrocytes were seeded on the top of the different scaffold hydrogels and after 7 days in culture cell tracking green assay was performed to check cell viability, and how the cells were able to penetrate into the hydrogel. Cell staining showed that cell adhesion and viability were higher in PA204 hydrogels than in PA391 hydrogels. The combination of PEGDA and PEG crosslinkers to create PA204-2 seemed to allow the cells to colonize the hydrogel in its full depth, whereas the use of only PEGDA crosslinker in PA204-1 limits the adhesion of chondrocytes to restricted areas. PA391-2 hydrogels, where PEGDA and PETA protein were used to create porous system, were more efficient to support chondrocytes adhesion and viability in comparison with PA391-1, where the number of attached and live cells was quite low. Moreover, the morphology of chondrocytes in PA391-1 hydrogels was not the typical chondrogenic form of polygonal and star-shaped cells but adopted a spindle-like appearance with increased size [Fig. 24]. These data might indicate that in PA391-1 hydrogel chondrocyte de-differentiate.

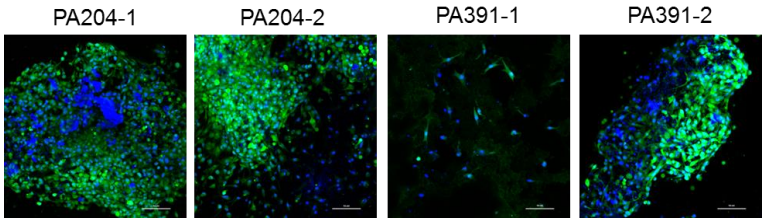

Fig. 24. Analysis of cell attachment, viability and competitive affinity of chondrocytes in hydrogels. Representative confocal laser scanning microscope images of primary human chondrocytes cultured in 3D hydrogels after seven days in culture. Live cells emitted green fluorescence (CTG). Magnification 20x. Scale bar = 100 µm.

These results demonstrated that the combination of different crosslinkers create a network that promotes cell adhesion, viability and maintained chondrocytes phenotype, and based on this premise, a new generation of polymers were made with different combination of crosslinkers.

Two new hydrogels were made, (i) PA204-2A that shared with PA204-2 the use of PEGDA, but a double concentration of PEG, and (ii) PA391-2A which combine three different crosslinkers, PEGDA, PEG and PETA [Fig. 25]. In order to create a high porous system, a replica of each polymer was lyophilized. Fresh isolated chondrocytes cultured in monolayer during seven days were seeded into the hydrogels, cultured for 21 days and cell viability and maintenance of chondrocyte phenotype were analyzed.

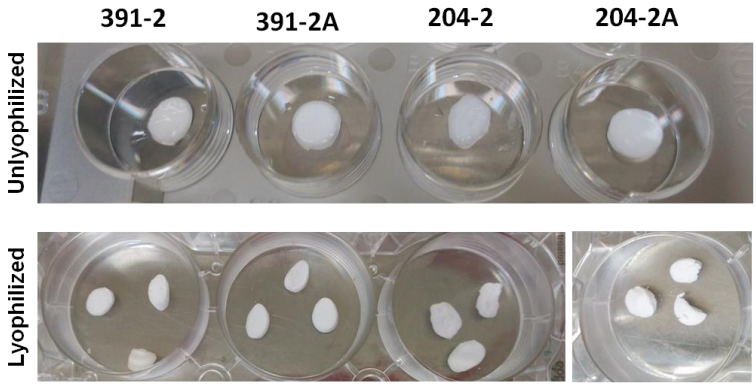

Fig. 25. Hydrogels. Representative images of different hydrogels before plated cells.

Live cells were stained with CTG, and propidium iodide (PI) was used to stain dead cells because PI is membrane impermeant and is excluded from viable cells. PA204-2 and PA391-2 unlyophilized hydrogel showed the best results with high confluence and cells with typical polygonal and star-shaped morphology after 21 days in culture. The rest of the polymer or did not promote proliferation, or displayed good viability and proliferation but, cells presented an increase in size with spindle-like fibroblast appearance [Fig. 26]) (Table 5).

Table 5. Cell viability after 21 days cultured on hydrogel. Hihg (+++), medium (++) and low (+) viability.

	PA204-2	PA204-2A	PA391-2	PA391-2A
Unlyophilized	+++	++	+++	+
Lyophilized	+++		+	+++

Immunofluorescence analysis for collagens expression was performed to check if cells maintained the chondrocyte phenotype after 21 days in culture. In general, PA204 hydrogels showed higher expression of Col II, the characteristic marker of mature chondrocyte phenotype, without expression of Col I (hypertrophic marker). In PA391 hydrogels the expression of Col I was predominant, showing minimal expression of Col II in PA391-2A lyophilized hydrogels [Fig. 26] [Table 6]. At this point, PA391 hydrogels were discarded, and subsequent studies were performed with PA204-2 unlyophilized hydrogels because presented better characteristics for chondrocytes culture.

Table 6. Chondrogenic phenotype after 21 days cultured on hydrogel. Col II (+) and Col I (x) expression. Hihg (+++), medium (++) and low (+) viability.

	PA204-2	PA204-2A	PA391-2	PA391-2A
Unlyophilized	+++	+	xxx	xxx
Lyophilized	++	+	+/xxx	xxx

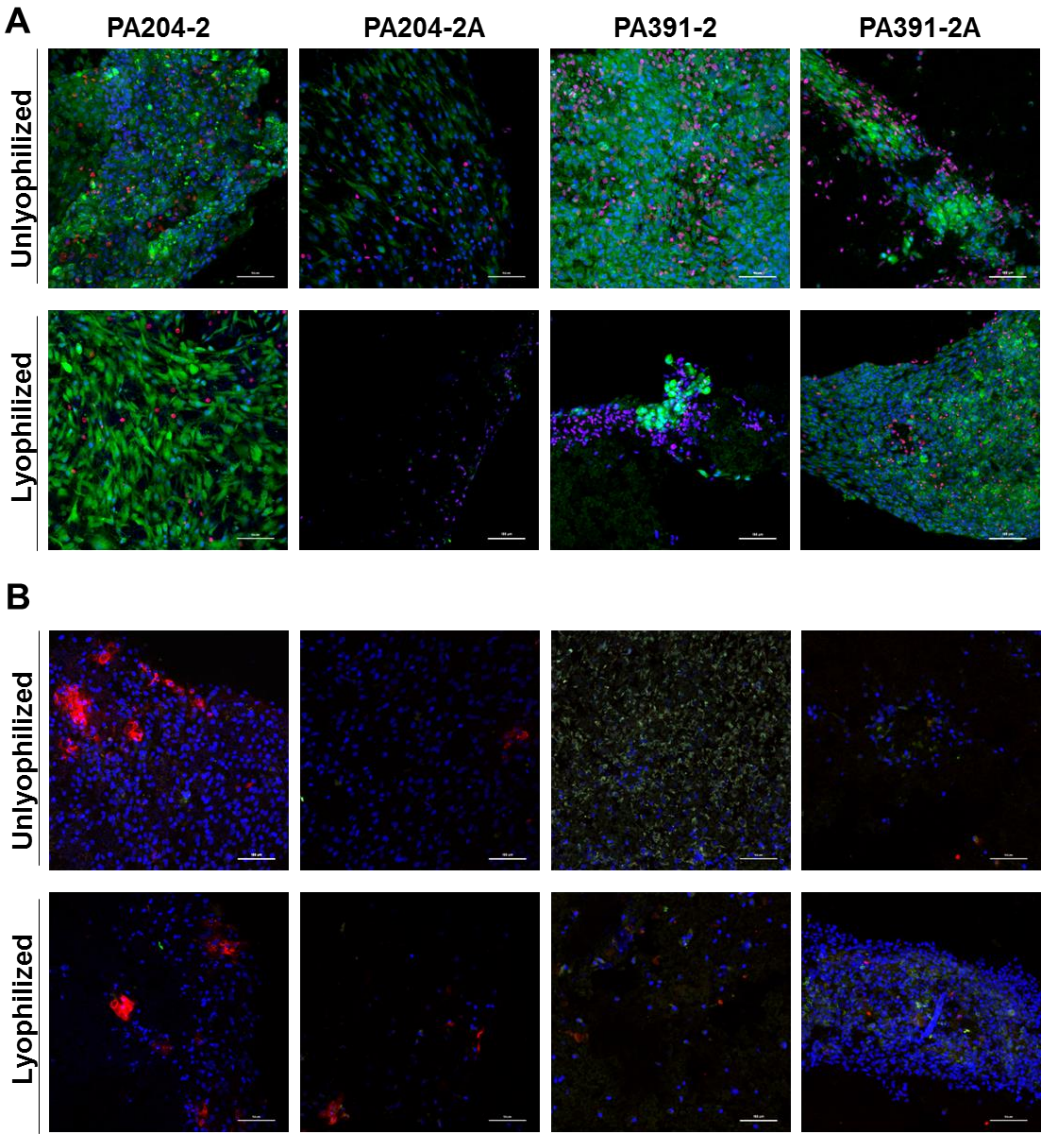

Fig. 26. Cell viability and chondrogenic markers after 21 days cultured on hydrogel. (A) Representative confocal laser scanning microscope images of primary human chondrocytes cultured in 3D hydrogels for 21 days. Live cells emitted green fluorescence (CTG) and dead cells were labeled by propidium iodide (PI) in red. Magnification 20x. Scale bar = 100 µm. **(B)** Cartilage matrix-related markers Col II (red) and Col I (green) staining of primary human chondrocytes cultured on hydrogels at 21 days. Magnification 20x. Scale bar = 100 µm (B).

2.1. PA204 unphyophilized hydrogel.

Histological analysis was performed in order to confirm the presence of cartilage-specific ECM components produced from chondrocytes [Fig. 27]. Hematoxilyn-Eosin staining of PA204-2 unlyophilized hydrogel sections revealed a porous system with complex internal structure more similar to native cartilage tissue that other variants of this hydrogel, with cells (dark purple) isolated in lacunae surrounded by dense ECM (pink). Masson-Trichrome and Alcian Blue revealed the presence of collagens fibers and proteoglycans (green and blue respectively) in PA204-2 unlyophilized hydrogel, whereas, in PA204-2 lyophilized hydrogel the presence of these components were less evident, being practically nonexistent in PA204-2A hydrogels.

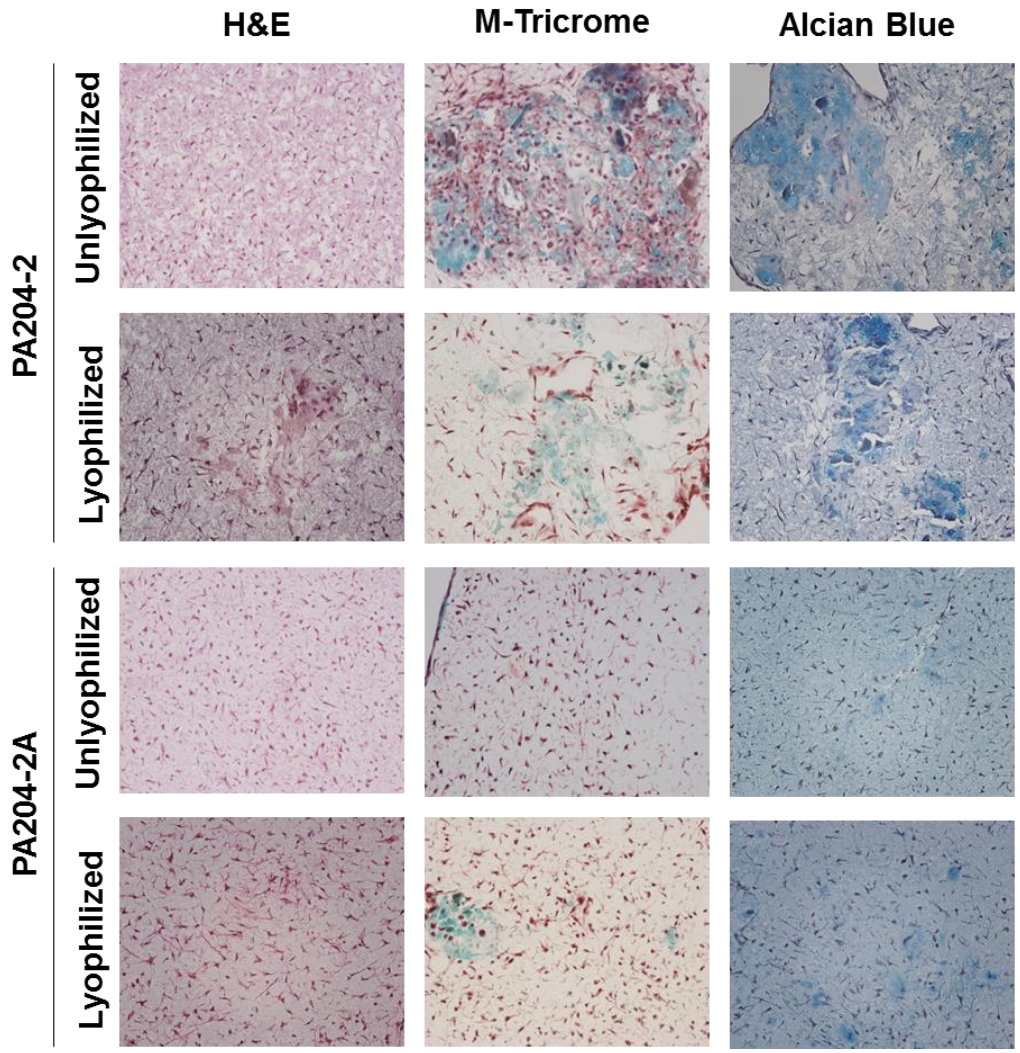

Fig. 27. Chondrogenic markers after 21 days in culture on PA204-2 and PA204-2A lyophilized and unlyophilized hydrogels. Histological staining of hydrogels sections shows that chondrocytes cultured on PA204-2 unlyophilized hydrogel secreted a cartilage like matrix as is

demonstrated by Hematoxilyn-Eosin, Masson´s Trichrome and Alcian Blue staining. Magnification 20x.

Taken together the previous results, PA204-2 unlyophilized hydrogel was selected as the most appropriate for chondrocytes culture, and for this reason later studies were focused on this hydrogel.

To gain furher information regarding the chondrocyte dispossition and the formation of ECM within the selected hydrogel, these samples were analyzed by ESEM. Results showed that PA204-2A unlyophilized hydrogel consisted in a microstructure with spherical shape interconnected to form a dense fibrillar structure. Hydrogel presented a microporus structure where pores with a diameter of about 20 µm were interconnected to form a macroporous system. ESEM analysis revealed a good adhesion and distribution of chondrocytes, attached on the hydrogel surface by filopodia and connected with each others. In addition, ECM secretion was observed, extended between cells and covering the surface of the hydrogel to form a continous blanket [Fig. 28].

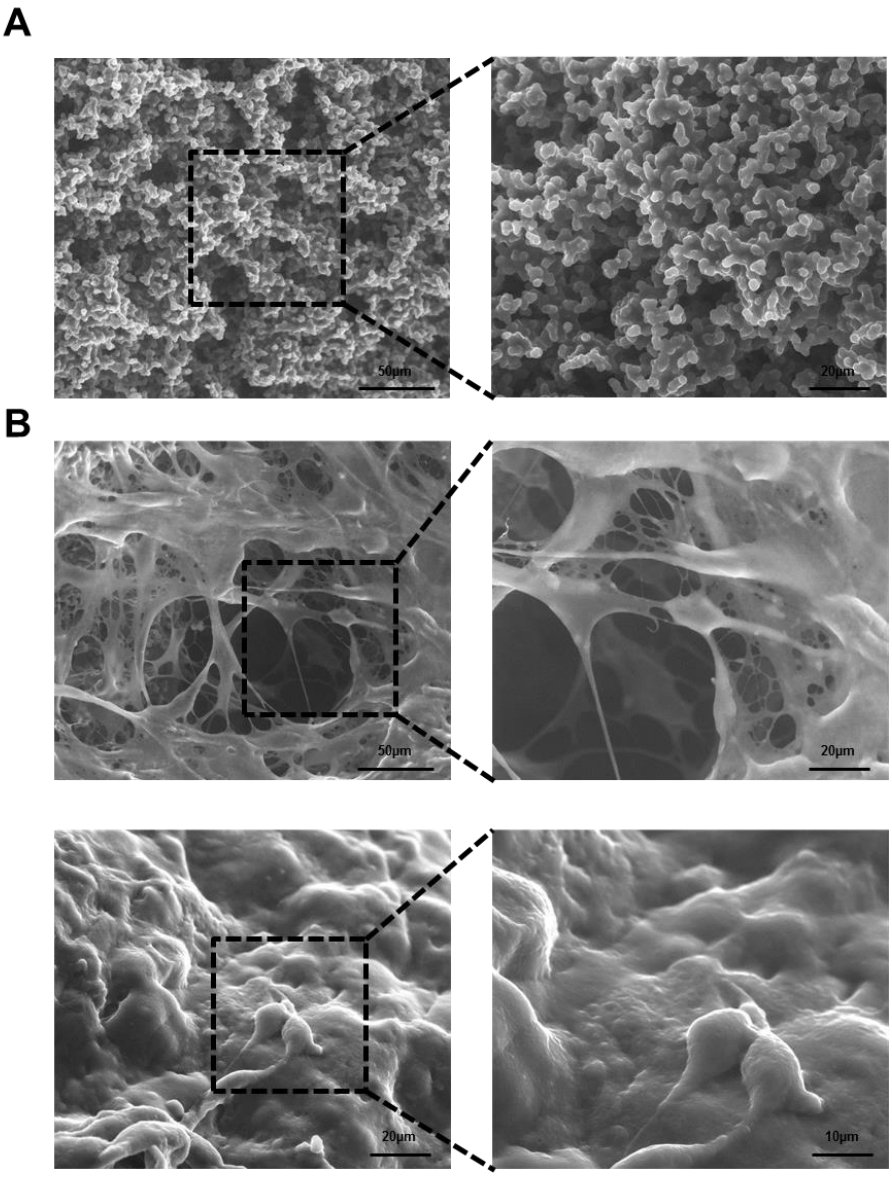

Fig. 28. Environmental scanning electron microscopy images of PA204-2 unlyophilized hydrogel at 21 days in culture . (A) PA204-2 unlyophilized hydrogel structure. **(B)** Human primary chondrocytes cultured on PA204-2 unlyophilized hydrogel showed a round shaped morphology and a rough surface, surrounded by dense matrix that cover the surface of the hydrogel.

Finally, quantitative analysis of the production of GAGs, and quantification of the chondrogenic gene expression was performed by qPCR. GAGs present on cartilage tissue engineered were solubilized through proteolitic digestion and, then, detected and quantificated by DMB colorimetric method. The total amount of GAGs produced by cells in PA204-2 unlyophilized hydrogel was in increase during 21 days in culture

[Fig. 29], fact that evidence the mature phenotype of chondrocytes, which secreted a ECM composed by GAGs accumulation.

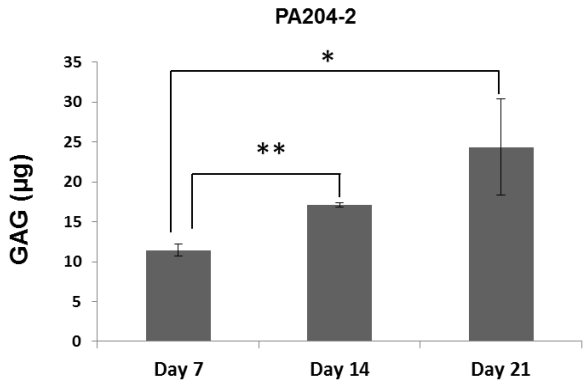

Fig. 29. Analysis of mature chondrocyte phenotype. Measurement of GAGs content of primary human chondrocytes cultured on PA204-2 unlyophilized hydrogels at 21 days. Significant differences * $p<0.05$ and ** $p<0.01$.

qPCR determinations in PA204-2 unlyophilized hydrogel showed an enhanced gene expression of the principal cartilage ECM components: Col II (2-fold), aggrecan (4-fold) and COMP, as well as, the expression of Sox9, the major transcription factor of chondrogenesis, increased to an average of 3-fold compared with fresly isolated chondrocyte cultured in monolayer for 7 days. The dedifferentiated and hypertrophic phenotype of chondrocytes cultured during long periods of time are characterized by the expression of Col I and Col X, and ECM of PA204-2 unlyophilized hydrogel did not display higher expression of these markers compared with mature chondrocyte in monolayer. Additionally, chondrocytes cultured in pellets system during 21 days was introduced in qPCR analysis as a 3D culture system control, and was very interesting that hydrogel showed similar expression in chondrogenic markers, but lower expression of hypertrophic markers Col I and Col X ($p<0.01$), demonstrating that PA204-2A unlyophilized hydrogel can improve the traditional 3D culture pellet system to maintain the chondrocyte phenotype [Fig. 30].

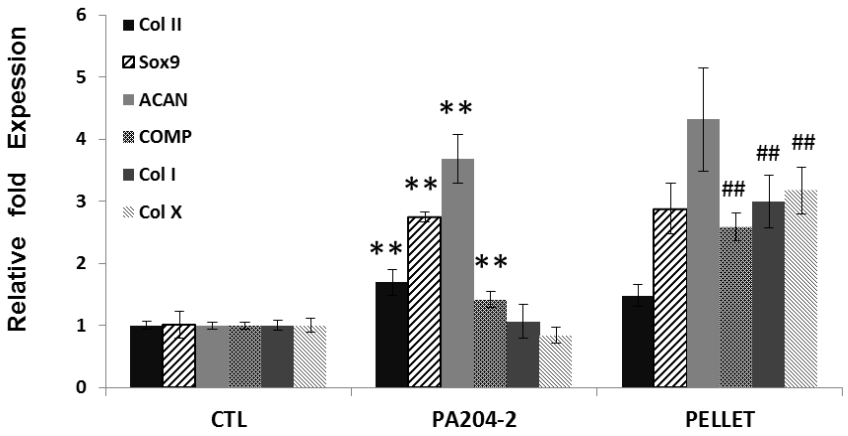

Fig. 30. Gene expression of chondrogenic markers. Real-time PCR analysis of selected chondrogenic markers of human chondrocytes cultured during 21 days on PA204-2 unlyophilized hydrogels. All the gene expressions were normalized with the values of 1 week chondrocytes in standard culture used as control (CTL). Statistical significant differences were found (** $p<0.01$) when compared gene expressions between CTL and PA204-2, and when compared PA204-2 and Pellet (## $p<0.01$).

3. Polymers solutions coating PCL scaffolds

There are different and numerous routes to construct 3D scaffolds adequate for cell culture and for cell TE, that gather the physico-chemical and mechanical suitable properties and that fits properly to the shape of the lesion to repair. Apart from to construct hydrogels, polymer solutions can be used to cover the surface of 3D constructs promoting cell growth or forming a proper contact surface between the tissue and the material.

Following this premise, PA204 and PA391 polymer solutions (2.0% w/v in acetic acid) were used to coat the surface of standard and commercial PCL scaffolds. Uncoated PCL scaffolds were used as control.

Cell viability was checked using CTG at the beginning of the experiment, and at 21 days. Images showed similar chondrocytes adhesion and viability at 48 hours between uncoated PCL scaffold, and PA204 and PA391-PCL scaffolds. Cell proliferation, cell

distribution and confluence were similar between the three samples after 21 days of culture [Fig. 31A]. Immunofluorescence analysis for Col II expression was performed to prove that after long periods of culture in polymer coated scaffolds, chondrocytes maintained their phenotype and could produce mature cartilage ECM. Results displayed that PCL scaffold and PA204-PCL scaffolds presented high expression and homogeneous distribution of Col II in the new ECM synthesized, whereas PA391-PCL scaffolds showed high expression but restricted to certain areas [Fig. 31B]. Finally, ESEM images evinced a dense extracellular matrix secretion that covers the surface, and good adhesion and homogenous distribution of chondrocytes throughout the entire scaffold in all cases [Fig. 31C].

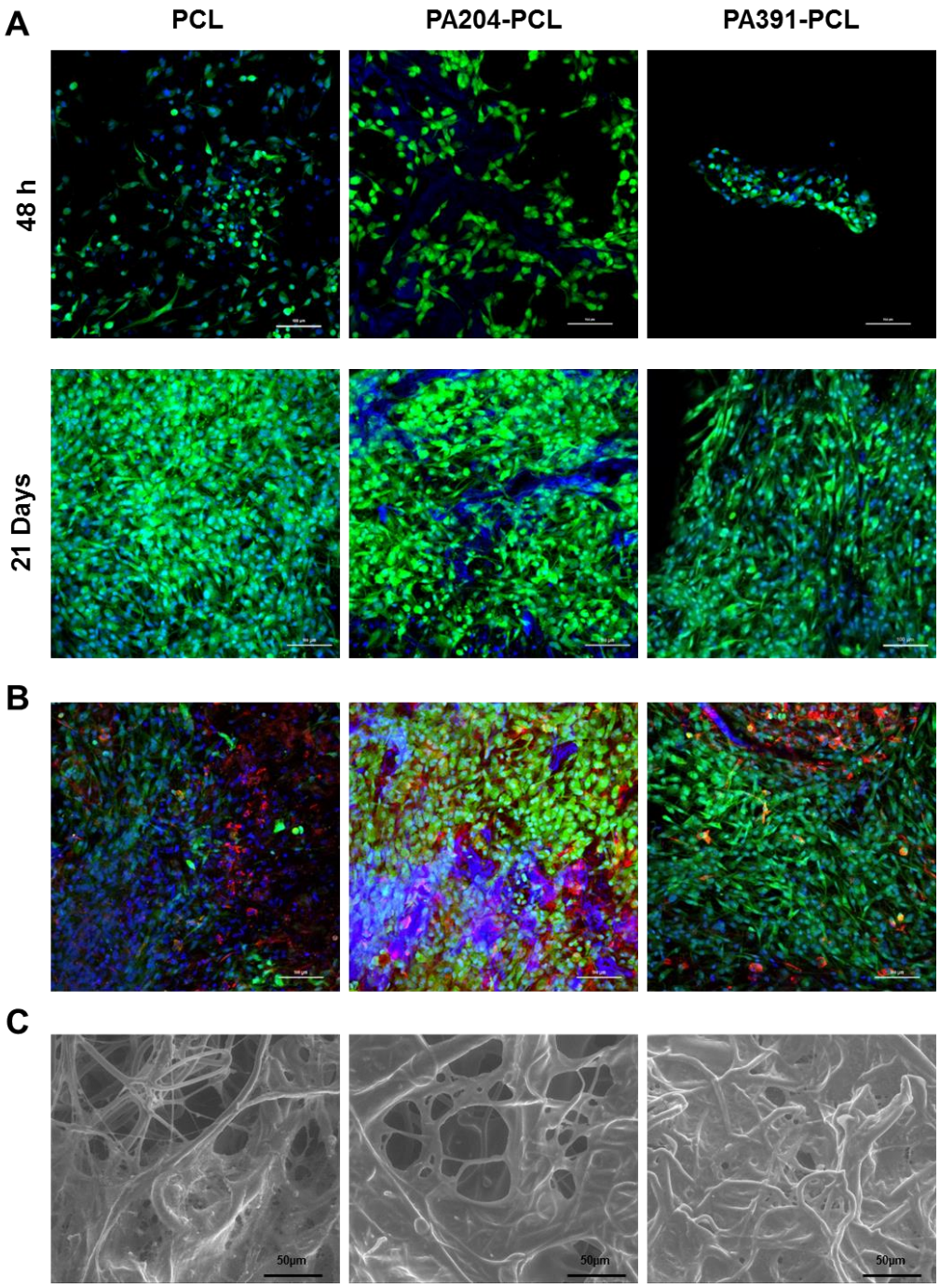

Fig. 31. Cell viability and chondrogenic markers after 21 days in culture on PCL-polymer coated scaffolds. (A) Confocal laser scanning microscopy images demonstrating the viability of chondrocytes cultured in uncoated and PCL-polymer coated scaffolds for 48 hours and 21 days using CTG (green). Magnification 20x. Scale bar = 100 µm. **(B)** Cartilage matrix marker Col II (red) and CTG (green) staining of chondrocytes cultured in uncoated and PCL-polymer coated scaffolds after 21 days in culture. Magnification 20x. Scale bar = 100 µm. **(C)** Environmental scanning electron microscopy images of primary human chondrocytes cultured on PCL and PCL-polymer coated scaffolds after 21 days in culture.

Discussion

Chondrocytes naturally reside embebed in a 3D network of ECM consisting in collagens (mainly Col II), glycosaminoglycans (mainly Aggrecan) and water. Unfortunately, when chondrocytes are cultured in a monolayer for long periods of time revert their phenotype to a dedifferentiated-hypertrophic state. The lost of differentiation is characterized by an increase in proliferation, morphological changes (fibroblast-like appearance), a decrease of Col II and proteglycans synthesis, and an increment in Col I expression (Schnabel et al. 2002).

It has been proved that chondrocytes cultured in a 3D system can retain their phenotype during prolonged culture, prevent the dedifferentiation process and produce a characteristic ECM (Takahashi et al. 2007) (Foldager et al. 2011). Moreover, it has been shown that 3D culture can reverts the phenotype of dedifferentiated chondrocytes (Barlic et al. 2008) (Benya & Shaffer 1982), and enhanced proliferation of mature chondrocytes compared with monolayer culture (Lin et al. 2009).

To provide chondrocytes with a suitable 3D environment different scaffolds have been designed. Scaffolds for TE should be non-toxic, should have a controlled degradation rate, and should be highly porous to allow the diffusion of nutrients and waste products. In addition, scaffolds should be made from materials that have suitable physical and mechanical properties, promote cell adhesion, viability, proliferation and ECM production (Chung & Burdick 2008). To find a proper material for constructing scaffolds that could be clinically applied, different polymers have been tested. To address the demand for finding the most favorable polymer for cartilage regeneration we have used here a powerful tool named polymer microarrays. This approach consists in a platform with hundreds of different polymers that allows a rapidly screening in parallel. Polymers are selected on base of cell attachment, cell growth, and even their capacity to induce cell differentiation (Hook et al. 2010)(Algahtani et al. 2014).

In the present study, we have used a high-throughput polymer microarray to screen and compare 380 different polymers, divided in 342 polyacrylates (PA) and 38 polyurethanes (PU), with the aim to discover new synthetic substrates for cartilage regeneration. Chondrocyte adhesion and cell viability was used to discriminate

between the polymers tested. Ten top polymers were selected, from which nine were PA and only one PU. Others studies has shown the feasibility of the use of PU polymers in soft TE, such as the creation of artificial heart valves, wound dressings, angioplasty balloons, ventricular assist devices (Stokes & Cobian 1982) and artificial skin (Dearman et al. 2014). In addition, PU polymers have been also employed to reproduce hard tissues (Tare et al. 2009) including cartilage tissue (Tsai et al. 2015) (Hung et al. 2014). Although PU polymers have been proved to be a suitable material with applications in TE, still these polymers present an important disadvantage for biomedical applications due to the toxic effect of its precursors. In order to solve this handicap, some investigators are trying to reduce precursor toxicity to enhance the biodegradable properties of the PU polymers (Ma 2008).

Acrylate-based polymers, PA, have also been widely used for the creation of hard tissues by TE approaches (Fisher et al. 2001) (Mountziaris et al. 2015). Furthermore, it has been shown that PA substrates could induce chondrogenesis in MSCs by itself, in the absence of any chondrogenic inductor (Glennon-Alty et al. 2013). In agreement with those findings, here we have proved that some of the top polymers chosen did increase synthesis of proteoglycans by ASCs.

When a cell interacts with a polymer the physicochemical characteristics of the synthetic matrix such as, charge, roughness, elasticity, surface topography, degradation and hydrophobicity, trigger different cellular pathways that can interfere in cell adhesion, cell proliferation and even the maintenance of a differentiated phenotype (Shin et al. 2003) (Ma 2008). Here we have shown that polymers tested provoked different responses on the chondrocytes, in terms of cell attachment. For instance, some polymers did not support cell growth and other, on the contrary, presented an enhance affinity for the chondrocytes.

Two polymers, PA204 and PA39, which proved to have fine characteristics that increased tissue formation, were selected and were used to create hydrogels. Hydrogels are 3D networks composed of crosslinked hydrophilic polymer chains, which can be cast into practically any shape, size, or form, and can retain a significant amount

of water. Their main characteristics, such as biocompatibility, flexibility in fabrication, variable composition and physicochemical and biological properties, convert hydrogels in a suitable tool for TE (Zhu & Marchant 2011). Specifically, hydrogels represent a good candidate to treat cartilage lesions, because it can exhibit similar mechanical, swelling, and lubricating behavior that articular cartilage. Moreover, hydrogel can promote and/or maintain a chondrogenic phenotype by creating a niche where cells are encapsulated, mimicking the native tissue (Seliktar 2012). Further, hydrogels can be delivered into the injured region by a simple injection, which implies several benefices. First, injected hydrogel can adopt the specific shape and size of the lesion; second, the hydrogel can be delivery in a minimally invasive manner minimizing irritation to the surrounding tissue, and third, hydrogel can be a suitable vehicle for cells and/or chondrogenesis inductors (Li et al. 2012).

Hydrogels are formed by the covalent crosslink of different hydrophilic polymer. The election of appropriate crosslinker is key, because can affect the mechanical and structural characteristics of the hydrogel, such as biocompability, visco-elasctic properties, consistency, porosity, and degradation behavior (Hennink & van Nostrum 2002). Here, we have created new hydrogels by binding polymers that were specifically selected to support chondrocyte growth, such as PA204 using PEGDA and PEG like crosslinkers.

The best results were obtained with the novo PA204-2 hydrogel, which displayed high cell adhesion, promoted cell viability at short and long time culture periods and enhanced cell proliferation. Furthermore, PA204-2 hydrogel was able to promote the formation of a cartilage tissue-like ECM with an increased expression of Col II, the principal marker of mature ECM in cartilage, and a decreased expression of Col I (Marlovits et al. 2004).

The pore size average, the pore size distribution, and the pore interconnections are important factors in a hydrogel (Hoffman 2002). In our study, chondrocytes presented an affinity for the smaller pores in unlyophilized hydrogel (Pina et al. 2015), growing covering this pores and secreting a high dense ECM that formed a homogenously

compact surface composed by collagens and proteoglycans, similar to native cartilage tissue.

Interestingly, after 21 days in culture, chondrocytes seeded into PA204-2 unlyophilized hydrogels maintained their native phenotype, secreting proteoglycans to construct a cartilage-like ECM. Further analysis by qPCR showed enhanced mRNA expression for chondrogenic markers (Coll II, ACAN, COMP and Sox9) when compared with chondrocytes that were freshly isolated and cultured in a monolayer system for 7 days. These results support the conclusion that PA204-2 hydrogel create the appropriate niche for chondrocyte growth and the formation of a cartilage like tissue. In fact, native cartilage ECM is composed mainly by Col II, aggrecan and COMP (Dell'Accio et al. 2001) (Hedbom et al. 1992). The transcription factor Sox9 is also highly expressed in chondroprogenitor cells and has a key role in chondrogenesis by means of promoting the expression of Col II, aggrecan and proteoglycans (Zhao et al. 1997) (Akiyama et al. 2002) (Lee et al. 2014). Furthermore, Sox9 acts as negative regulator of cartilage vascularization, endochondral ossification and osteogenic differentiation (Hattori et al. 2010) (Liao et al. 2014). Another important finding was that the expression of the fibrotic and hypertrophy markers Col I and Col X (Gu et al. 2014) was not enhanced in the "novo tissue" supported by the PA204-2 unlyophilized hydrogel. Summarizing, PA204-2 unlyophilized hydrogel is an optimal 3D culture system that promotes chondrocyte survival and the production of a cartilage-like ECM.

To enhance the adhesion and proliferative potential of cells, coating culture surface with a material has been extensively adopted (Yashiki et al. 2001). Numerous studies had demonstrated the efficacy of coated surfaces in the culture of chondrocytes; for instance, the use of poly lactic acid promote cytocompatibility and improve the characteristics of this polymer to the surface that has been coated (Zhu et al. 2004). Other example is the use of chitosan and galectin-1 to cover polylactideco-glycolide scaffolds (Chen et al. 2010). In this sense, we coated PCL scaffolds with PA204 and PA391 polymers, and after 21 days in culture the proliferation and phenotype of chondrocytes were evaluated. The ECM in PA204-PCL and uncoated PCL scaffolds was characterized by the production of Col II. It was proved that chondrocytes cultured in

PA204-PCL during 21 days did not dedifferentiate but no differences were found between coated and un-coated PCL scaffolds. Others have found that the coating of PCL scaffolds with hyaluronic acid could improve the composition of ECM secreted by chondrocytes cultured under deddiferentiation conditions (Lebourg et al. 2013).

In the present study, high-throughput microarray procedure was used to screen a library of PA and PU polymers to identify polymers with potential use for TE to treat chondral lesions. Nine PA and one PU polymers were identified as suitable polymers for the adhesion, viability and proliferation of chondrocytes. Two polymers were chosen and used to construct hydrogel scaffolds. New hydrogel scaffolds proved to support cartilage-like tissue formation and might represent a promising candidate for cartilage TE in the clinic.

CONCLUSIONS

1. Nodal/BMP2 (NB260) and Activin/BMP2 (AB235) chimeric ligands can induce efficient differentiation towards chondrocytic phenotype of adipose derived stem cells obtained from the knee of OA patients (IFPSCs) and from liposuctions (ASCs).

2. AB235 chimera leads chondrogenic differentiation more efficiently than BMP2, incrementing chondrogenic markers and maintaining or decreasing hypertrophic markers in adipose derived stem cells.

3. ASCs cultured under 3D pellet system in combination with chondrogenic inductors treatment (AB235, NB260 and BMP2) showed higher expression of chondrogenic and hypertrophic markers than IFPSCs. Moreover, ASCs pellets can integrate into the surrounding tissue in the *in vivo* model more efficiently than IFPSCs pellets.

4. AB235 induces redifferentiation of functional OA patient-derived dedifferentiated chondrocytes in 3D pellet culture system. Extracellular matrix secreted by redifferentiated chondrocytes was similar to native cartilage tissue. Moreover, AB235-treated pellets can integrate into the surrounding tissue *in vivo*.

5. Only 3D pellet culture system is not sufficient stimulation to induce redifferentiation of dedifferentiated chondrocytes, as showed the poor expression of chondrogenic markers, and instability in the *in vivo* implantation.

6. Polymers based in combinations of polyurethanes and polyacrylated monomers have good intrinsic properties for chondrocytes adhesion, viability and proliferation.

7. Unlyophilized hydrogel based in PA204 polyacrylate polymer with PEGDA and PEG as crosslinker creates an environment that encourages the maintenance of chondrocyte phenotype after long periods in culture, avoid the dedifferentiation and hypertrophic state. This polyacrylated based hydrogel constitute a promising candidate for cartilage tissue engineering.

8. PA204-coated polycaprolactone scaffolds maintain the chondrocyte phenotype and secretion of dense extracellular matrix after long periods in culture, as the goal standard polycaprolactone polymer.

9. The combination of novel chimeric ligands with polyacrylate polymers has clinical potential for tissue engineering focused to treat and regenerate lesions in cartilage.

CONCLUSIONES

1. Los ligandos quiméricos Nodal/BMP2 (NB260) y Activin A/BMP2 (AB235) pueden inducir la diferenciación eficiente hacia el fenotipo condrocítico de células madre derivadas de tejido adiposo obtenidas de la grasa de la rodilla de pacientes con osteoartritis (IFPSCs) y de liposucciones (ASC).

2. AB235 conduce hacia una diferenciación condrogénica de forma más eficiente que BMP2, incrementando marcadores condrogénicos y manteniendo o disminuyendo marcadores de hipertrofia en las células madre derivadas de tejido adiposo.

3. ASC cultivadas en 3D en pellets celulares en combinación con la inducción condrogénica por factores de crecimiento (AB235, NB260 y BMP2), mostraron una mayor expresión de marcadores condrogénicos y de hipertrofia que IFPSCs. Por otra parte, los pellets celulares de ASC pueden integrarse en el tejido circundante en el modelo *in vivo* de manera más eficiente que los pellets IFPSCs.

4. AB235 induce re-diferenciación de condrocitos desdiferenciados obtenidos de pacientes con osteoartritis mediante el sistema de cultivo en 3D de pellets celulares. La matriz extracelular secretada por los condrocitos re-diferenciados fue similar al tejido de cartílago nativo. Por otra parte, los pellets tratados con AB235 pueden integrarse en el tejido circundante *in vivo*.

5. El sistema de cultivo en pellets celulares por sí solo no es suficiente para inducir la estimulación de re-diferenciación de los condrocitos desdiferenciados, como mostró la pobre expresión de marcadores condrogénicos, y la inestabilidad de la implantación *in vivo*.

6. Los polímeros basados en combinaciones de monómeros de poliuretanos y poliacrilatos tienen buenas propiedades intrínsecas para la adhesión, viabilidad y proliferación de condrocitos.

7. Hydrogel sin liofilizar creado a partir del polímero de poliacrilato PA204 con PEGDA y PEG como crosslinkers crea un entorno que fomenta el mantenimiento del fenotipo de

los condrocitos durante largos períodos de cultivo, evitando la desdiferenciación y la hipertrofia. Este hidrogel basado en poliacrilato constituye un candidato prometedor para la ingeniería de tejidos de cartílago.

8. Scaffolds de policaprolactona recubiertos con el polímero PA204 mantienen el fenotipo de los condrocitos y la secreción de densa matriz extracelular después de largos períodos de cultivo, como el scaffold de policaprolactona estándar.

9. La combinación de los novedosos ligandos quiméricos con polímeros de poliacrilato tiene potencial clínico para la ingienería tisular enfocada a la regeneración y tratamiento de lesiones de cartílago.

ABREVIATIONS LIST

3D: Three-Dimensional

AB: Alcian Blue

AB235: Activin A/BMP2

ACAN: Aggrecan

ACI: Autologous Chondrocytes Implantation

ADAM: A Disintegrin-like and Metalloproteinase-like Domain

ADAMTS: A Disintegrin-like and Metalloproteinase-like Domain Thrombospondin Domain

APS: Ammonium persulfate

AR: Alizarin Red

BMP: Bone Morphogenetic Protein

cDNA: complementary DNA

Col I: Collagen I

Col II: Collagen II

Col IV: Collagen III

Col X: Collagen X

COMP: Cartilage Oligomeric Matrix Protein

CT: Cycle Threshold

CTG: Celltracker Green

DAPI: 4',6-Diamidino-2-Phenylindole Dihydrochloride

DMB: 1,9-Dimethylmethylene Blue

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic Acid

EBs: Embryonic Bodies

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic Acid

ESC: Embryonic Stem Cells

ESEM: Environmental Scanning Electron Microscope

FACS: Fluorescence-activated Cell Sorting

FBS: Fetal Bovine Serum

FGF: Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor Receptor

FITC: Fluorescein Isothiocyanate

GAGs: Glycosaminoglycans

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GDF: Growth Differentiation Factor

H&E: Hematoxylin and Eosin

HA: Hyaluronic Acid

HBSS: Hank's Balanced Salt Solution

IFPSCs: Infrapatellar fat pad-derived Stem Cells

IGF: Insulin-like Growth Factor

iPSCs: induced Pluripotent Stem Cells

ISCT: International Society for Cellular Therapy

MACI: Matrix-Induced Autologous Chondrocyte Implantation

MMPs: Matrix Metalloproteinases

mRNA: messenger RNA

MS: Masson´s-Trichrome

MSC: Mesenchymal Stem Cells

NB260: Nodal/MP2

NMP: 1-Methyl-2-pyrrolidinone

OA: Osteoarthritis

PA: Polyacrylate

PBS: Phosphate Buffered Saline

PCL: Poly(epsilon-Caprolactone)

PE: Phycoerythrin

PEG: Poly(Ethylene Glycol)

PEGDA: Poly(Ethylene Glycol) Diacrylate

PETA: Pentaerythritol Triacrylate

PFA: Paraformaldehyde

PGA: Pol(Glycolic Acid)

PI: Propidium Iodide

PLA: Poly(Lactic Acid)

PLGA: Poly(Lactic-co-Glycolic Acid)

PTHrP: Parathyroid Hormone-Related Peptide

PU: Polyurethane

qPCR: Quantitative Polymerase Chain Reaction

RASCH: Random Assembly of Segmental Chimera and Heteromers

RNA: Ribonucleic Acid

RT: Room Temperature

RT-PCR: Reverse Transcription Polymerase Chain Reaction

TB: Toluidine Blue

TE: Tissue Engineering

TEMED: Tetramethyl-ethylenediamine

TGF-β: Transforming Growth Factor β

TNF: Tumor Necrosis Factor

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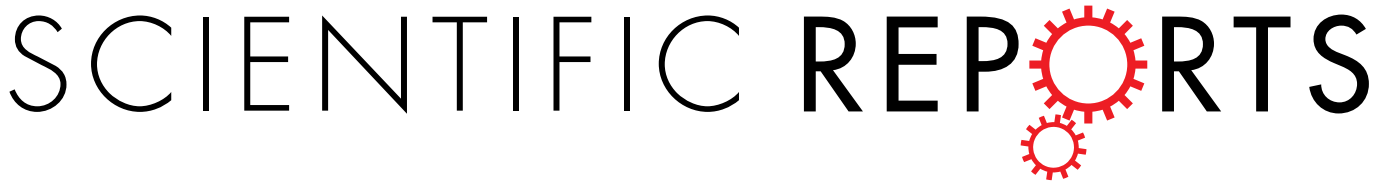

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Activin A/BMP2 chimera AB235 drives efficient redifferentiation of long term cultured autologous chondrocytes

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Autologous chondrocyte implantation (ACI) depends on the quality and quantity of implanted cells and is hindered by the fact that chondrocytes cultured for long periods of time undergo dedifferentiation. Here we have developed a reproducible and efficient chondrogenic protocol to redifferentiate chondrocytes isolated from osteoarthritis (OA) patients. We used morphological, histological and immunological analysis together with a RT-PCR detection of collagen I and collagen II gene expression to show that chondrocytes isolated from articular cartilage biopsies of patients and subjected to long-term culture undergo dedifferentiation and that these cells can be redifferentiated following treatment with the chimeric Activin A/BMP2 ligand AB235. Examination of AB235-treated cell pellets in both *in vitro* and *in vivo* experiments revealed that redifferentiated chondrocytes synthesized a cartilage-specific extracellular matrix (ECM), primarily consisting of vertically-orientated collagen fibres and cartilage-specific proteoglycans. AB235-treated cell pellets also integrated into the surrounding subcutaneous tissue following transplantation in mice as demonstrated by their dramatic increase in size while non-treated control pellets disintegrated upon transplantation. Thus, our findings describe an effective protocol for the promotion of redifferentiation of autologous chondrocytes obtained from OA patients and the formation of a cartilage-like ECM that can integrate into the surrounding tissue *in vivo*.

The high incidence of chondral lesions and the lack of a definitive treatment, particularly due to the intrinsic characteristics of the cartilage tissue, have an important impact on the health services systems in developed countries. Among approaches to treat articular cartilage lesions, autologous chondrocyte implantation (ACI) has been established as a good clinical therapeutic strategy for treating small injuries[1]. Nevertheless, this procedure has important limitations including the restricted number of chondrocytes

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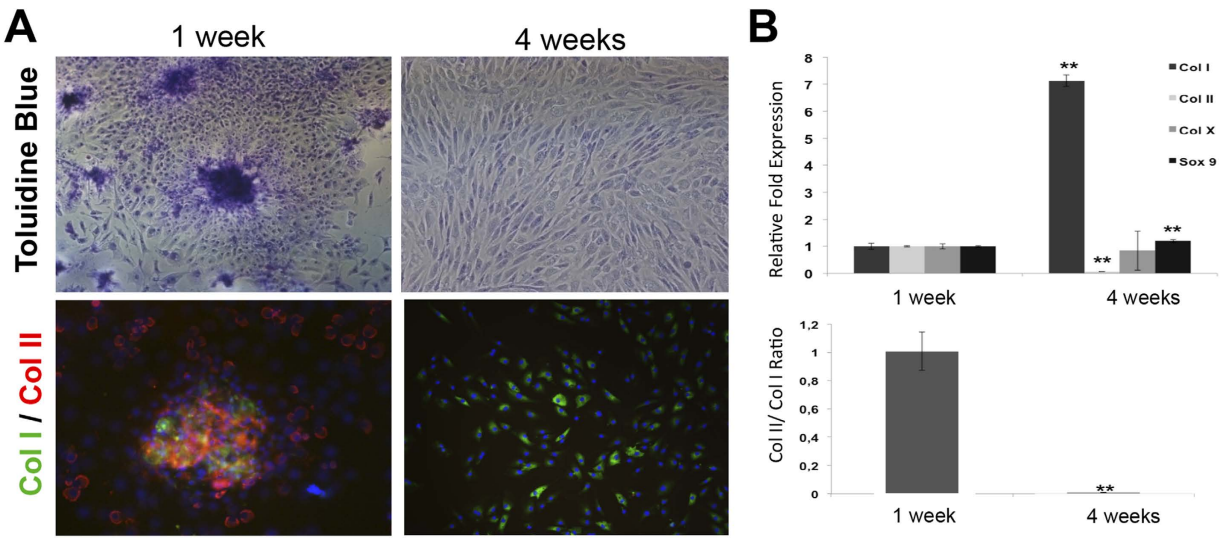

Figure 1. Dedifferentiation of chondrocyes grown in monolayer culture. (**A**) Chondrocytes cultured for 1 week or 4 weeks were stained with Toluidine Blue and immunolabeled for Col I (green) and Col II (red). (**B**) Real-time PCR analysis of selected chondrogenic markers after 4 weeks of monolayer cell culture. The bottom graphic shows the ratio of Col II versus Col I expression during the process of differentiation. **Statistical significance indicated ($p < 0.01$).

that can be isolated from a patient biopsy. In fact, in order to increase the number of cells, freshly isolated chondrocytes are cultured and expanded *in vitro*. However, this leads to the problem of loss of the chondrocyte phenotype due to cell dedifferentiation that occurs during prolonged monolayer culture[2]. This dedifferentiation causes chondrocytes to lose their round shape and become flattened fibroblast-like cells with an increased proliferative capacity and is accompanied by changes in gene expression and surface markers including decreased Col II and aggrecan and increased levels of Col I, Col X and COMP[3]. This loss of the differentiated chondrocyte phenotype upon culture *in vitro* represents a major disadvantage of the ACI technique because a decreased ratio of collagen type II/I results in production of an extracellular matrix typical of fibrotic tissue that might compromise cartilage regeneration[4].

Since the success of ACI depends on the number and quality of the cells to be implanted into the chondral lesion, approaches to revert dedifferentiation, called redifferentiation, are being investigated. In this respect, some studies have focused on using 3D cultures[5] or growth factors, such as members of the TGF-β superfamily including bone morphogenetic proteins (BMPs)[6,7].

BMPs and activins are structurally related members of the TGF-β superfamily of ligands but signal through different pairs of receptors[8]. Activin A exhibits very high affinity for its type II receptors, ActRII and ActRIIB, whereas BMP2 possesses low affinity for these receptors and higher affinity for its type I receptors. Since Activin A and BMP2 bind different type I receptors they activate distinct signalling pathways, i.e. Activin A activates SMAD2/3 transcription factors while BMP2 activates SMAD1/5/8 transcription factors[9]. We previously reported the creation of chimeric ligands based on systematic swapping of BMP2 and Activin-A sequences using a strategy termed Random Assembly of Segmental Chimera and Heteromers (RASCH)[10]. We found that one of these chimeras, AB235, significantly promotes chondrogenic differentiation of adipose-derived stem cells[11].

Here we demonstrate that AB235 effectively induces redifferentiation of functional osteoarthritis (OA) patient-derived dedifferentiated chondrocytes. Our results establish a novel protocol for re-establishing and maintaining the mature chondrocyte phenotype when cells are cultured for extended periods *in vitro*. This approach has the potential to facilitate and enhance treatment of cartilage-related injuries using ACI.

Results

Chondrocyte dedifferentiation upon monolayer culture. Freshly isolated chondrocytes were grown in monolayer culture up to passage 6 (P6) to ensure a complete dedifferentiation. Dedifferentiation was evident as soon as P3, when the proliferation of the cells increased and some chondrocytes started to change morphology and adopt a spindle-like fibroblastic shape. After 4 weeks all cells had increased in size and adopted a fibroblast-like appearance Fig. 1(A), while control chondrocytes cultured for only 7 days retained a typical polygonal and star-shaped morphology. Toluidine Blue staining, which reflects synthesis of glycosaminoglycans (GAGs), was clearly decreased in cell monolayers at P6 relative to control chondrocytes Fig. 1(A). Further, chondrocytes cultured for 4 weeks showed decreased Col II

expression (red staining) and increased Col I expression (green staining) while control chondrocytes showed the opposite Fig. 1(A).

Finally, we evaluated the expression of selected chondrogenic markers by qRT-PCR Fig. 1(B) and found that chondrocytes at P6 have increased expression of Col I ($p < 0.01$) and Col X and decreased expression of Col II ($p < 0.01$) when compared with control cells Fig. 1(B). A slight increase of Sox 9 expression ($p < 0.01$) was also detected specifically in long-term cultured chondrocytes. Furthermore, a significant decrease of the Col II/Col I ratio ($p < 0.01$) was observed in chondrocytes at P6 when compared to the ratio in the control cells. Together, these results indicate chondrocytes cultured in monolayer for four weeks adopt a dedifferentiated, fibroblast-like phenotype.

AB235 promotes redifferentiation of dedifferentiated chondrocytes. We tested if the chimeric AB235 ligand could induce redifferentiation of chondrocytes that have lost their differentiated phenotype following extended monolayer culture. Dedifferentiated cells were cultured for 4 weeks under pellet-forming conditions in chondrogenic medium either containing or lacking the AB235 chimeric ligand and then analyzed using histological and immunofluorescence probes. Pellets cultured in AB235-containing medium showed a noticeable increase in size relative to untreated controls. In addition, the AB235-induced pellets had a consistency and appearance more similar to native cartilage tissue Fig. 2(A). We compared the internal structure of the AB235-treated and non-treated pellets and found that the ECM of treated pellets was also more similar to that of native cartilage tissue Fig. 2(B). Pellet sections stained for H&E showed that AB235 induced the formation of a complex cellular organization with cells embedded in lacunae surrounded by ECM (staining in pale pink), again resembling native cartilage tissue, while ECM was not visible when cells were cultured under control conditions. Masson-Trichrome staining revealed collagen-specific staining (green) in the ECM of AB235-treated pellet sections that was similar to the staining of the native tissue but not visible in control pellets. Cartilage specific proteoglycans were also clearly more apparent in AB235-treated pellets than in the control as assessed by Alcian Blue and Toluidine Blue assays pellets (blue and purple respectively).

Further, Col II, Sox 9 and Aggrecan were expressed in the AB235-induced pellets at significantly higher levels than in the control pellets that showed weak and diffuse staining for these markers of chondrocyte differentiation Fig. 2(C). The patterned arrangement of Col II and Aggrecan fibres is striking and in stark contrast with the homogeneous distribution of this protein in control pellet sections.

Finally, quantitative image analysis was performed using ImageJ. For histological and immufluorescence images, the staining of AB235-treated pellets was significantly higher ($p < 0.01$) in all cases when compared with control pellets Fig 3.Together, these data indicate that AB235 promotes re-establishment of the chondrocytic phenotype in cells that lose this phenotype following extended culture periods *in vitro*.

Chondrocytes redifferentiated by AB235 *in vitro* promote cartilage integration upon transplantation in mice. We tested whether chondrocytes redifferentiated as a pellet *in vitro* are capable of maintaining their 3D structure after being transplanted into mice. Figure 4A shows a schematic representation of the experimental design we employed. Pellets obtained after 6 weeks of culture in the presence or absence of AB235 were transplanted into subcutaneous tissue on the flanks of inmunodeficient mice and then harvested 4 weeks later for histological and immunofluorescence analysis.

We find that the control pellets are completely absorbed by the surrounding mouse tissue and could not be recovered for histological and immunofluorescence analysis. By contrast, AB235-treated pellets displayed a dramatic increase in size over the 4 week period demonstrating that the graft was well tolerated by the organism Fig. 4(A). Histological analysis reveals that the ECM synthesized by redifferentiated chondrocytes is cartilage-specific pericellular matrix consisting primarily of vertically-oriented collagen fibres and proteoglycans Fig. 4(B). Integration of the AB235-treated pellet into the surrounding mouse tissue is demonstrated by the formation of *novo* tissue around the pellet that can be seen in the H&E stained section as shown in Fig. 4B. Finally, our immunofluorescence assay for collagens I and X shows that AB235 treatment does not induce fibrotic or hypertrophic cartilage formation Fig. 4(C,D). On the other hand, collagen II and Sox 9 markers were highly expressed with an arranged Col II distribution typical of a structured ECM and with Sox 9 localized in both the nucleus and cytoplasm Fig. 4(E,F). Finally, our results showed that Col X was almost undetectable in AB235-treated cells.

Discussion

Autologous chondrocytes are suitable for cell therapy strategies directed to repair cartilage tissue degeneration or damage. However, these strategies are hampered by the fact that chondrocytes undergo dedifferentiation when they are grown in monolayer culture for prolonged periods[2]. To overcome this limitation, we developed a robust protocol to redifferentiate chondrocytes that have undergone such *in vitro* culture-induced dedifferentiation.

Chondrocyte dedifferentiation has been described to occur as soon as 4–10 days after cells are plated in a monolayer[4]. We ensured full dedifferentiation toward a fibroblastic phenotype by growing chondrocytes in monolayer culture over a period of 4 weeks. Morphological, histological and immunological analysis together with real-time PCR measurement of Col I and Col II gene expression confirmed the

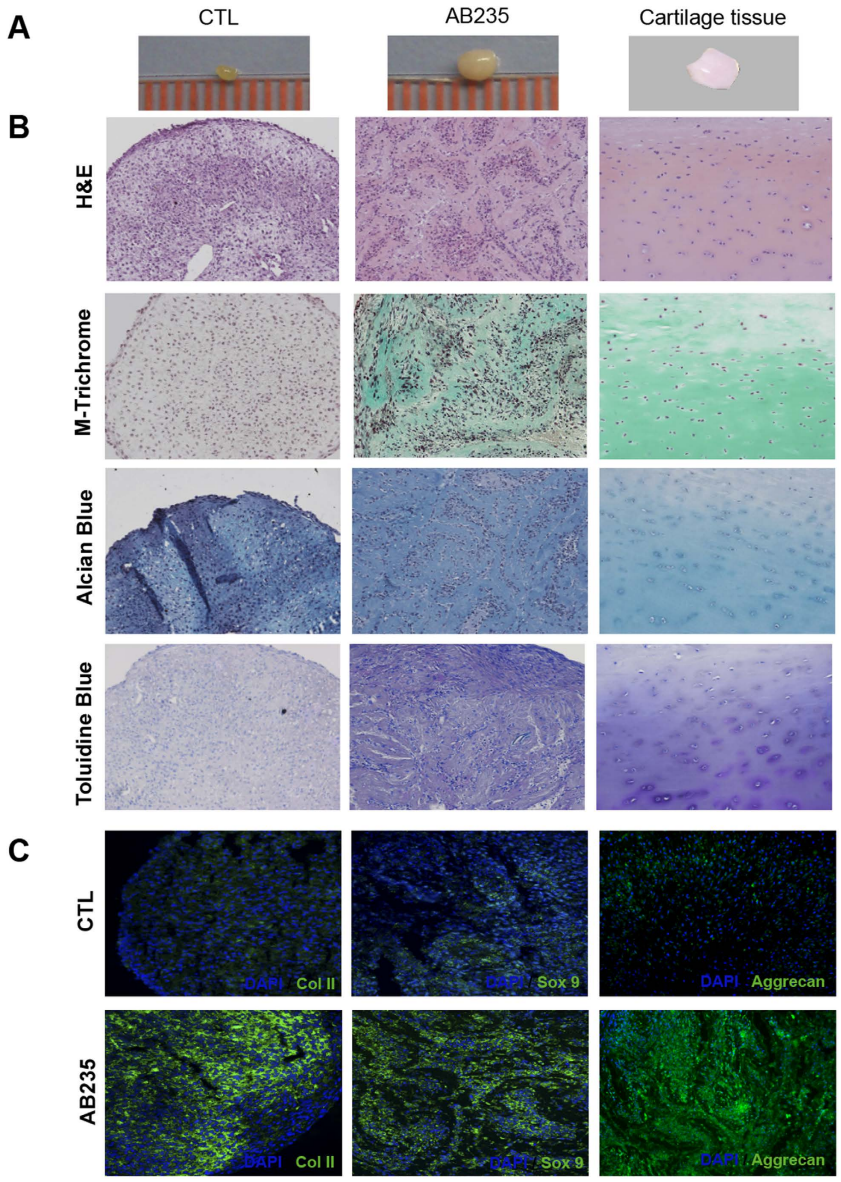

Figure 2. AB235 induces chondrocyte redifferentation *in vitro*. (**A**) Representative images of dedifferentiated chondrocytes cultured in a pellet system for 6 weeks in the absence of treatment (CTL) or treated with 10 ng/ml of the chimeric ligand (AB235) are compared with an image of native cartilage tissue. (**B**) Histological staining of sections of the pellets from **(A)** shows the acquisition of a cartilage like matrix resulting from AB235 treatment. (**C**) Merged images of pellet sections inmunostained with Col II, Sox9 and Aggrecan antibodies (green channel) and cell nuclei labelled with DAPI (blue channel) demonstrate that AB235 treatment increases of chondrogenic marker expression. Original magnification: 20× for all panels.

complete dedifferentiation of chondrocytes over this 4 week period in a manner that is in agreement with prior findings[13].

We have previously reported the creation of chimeric ligands that combine BMP2 and Activin-A sequences[10]. We subsequently confirmed the chondrogenic potential of Activin- and Nodal-like chimeras including AB235 in directing chondrogenic differentiation of adipose derived stem cells[11,14]. In the present study, we specifically hypothesized that AB235 reverts dedifferentiated chondrocytes back to their previous, fully differentiated chondrocytic state. We tested this hypothesis by culturing dedifferentiated chondrocytes under 3D conditions. It is known that cell-to-cell contact promotes chondrogenic differentiation and 3D culturing has been used before to induce redifferentiation of monolayer-expanded autologous chondrocytes[5]. However, our results showed that 3D culturing is not sufficient *per se* to redifferentiate chondrocytes as proven by the small size and lack of proper tissue organization found in the control pellets. On the other hand, pellets cultured with AB235 produced a cartilaginous matrix comparable to the ECM found in native cartilage tissue. These findings suggest that the AB235 ligand interacts

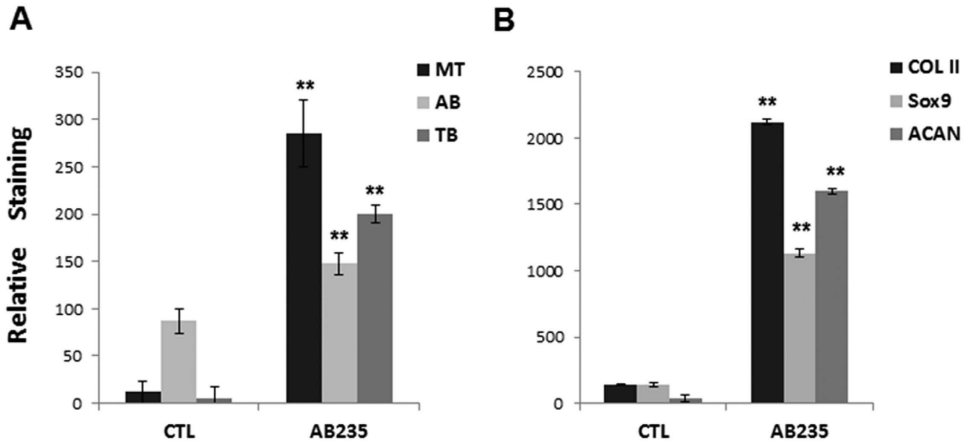

Figure 3. Quantitative image analysis. Graphical representation of the quantification of histological (**A**) and immunofluorescence (**B**) staining of control and AB235-treated pellet sections. MT: Masson-Trichrome; AB: Alcian Blue; TB: Toluidine Blue; ACAN: Aggrecan. **Statistical significance indicated ($p < 0.01$).

with Activin/BMP receptors in a way that promotes the reversion toward a chondrocytic phenotype in a manner concordant with what previous studies using BMP2 have shown[15,16]. In fact, the enhanced chondrogenic differentiation of human adipose derived stem cells (hASCs) treated with AB235 relative to that of cells treated with BMP2 suggests that this chimeric ligand signals more efficiently than BMP2 through type I and type II receptors that mediate cartilage maturation[11].

Furthermore, we demonstrate that AB235-induced cartilage integrates into the subcutaneous tissue upon transplantation into the flanks of immune compromised mice and that the structural cartilage-like complexity of the ECM in AB235-treated pellets strongly resembles native cartilage. By contrast, non-treated control pellets were absorbed by the surrounding tissue and could not be recovered for examination, in agreement with others studies that have showed that dedifferentiated chondrocytes failed to form cartilage tissue *in vivo*[17,18]. Interestingly, Activin A has been shown to be an inhibitor of matrix metalloproteinase 3 and to block the degradation of the ECM by this enzyme[19] raising the possibility that AB235, which utilizes the activin pathway, may exert similar effects.

From the present study we can conclude that the combination of sequences of BMP2 and Activin-A present in AB235 result in a ligand that increases the expression of chondrogenic markers of dedifferentiated chondrocytes. Others studies have shown that BMP2 upregulates chondrogenic gene expression of human articular chondrocytes expanded *in vitro*[20] and, therefore, comparing the chondrogenic potential of BMP2 versus AB235 would be of great interest for future studies.

In conclusion, we describe an effective protocol for redifferentiation of autologous chondrocytes obtained from OA patients and the formation of a cartilage-like ECM that can integrate into the surrounding tissue *in vivo*. Future work will include assessment of the tumour-forming potential of AB235-treated cells in order to determine if the procedure can be translate to the clinic. Since the success of cell therapies for cartilage injury depends on the quality and quantity of the implanted cells, our protocol may have significant potential for clinical applications.

Material and Methods

Patients. Articular cartilage was obtained from from 4 female and 4 male patients with knee osteoarthritis during joint replacement surgery at the University Hospital of Málaga, Spain, according to the guidelines of the University Hospital of Malaga (Table S1). Informed patient consent was obtained for all samples used in this study and samples were collected in accordance with the Research Ethics Committees of the University Hospital of Malaga, "Virgen de la Victoria" and University of Granada (ES180870000164). Average patient age at resection for females and males was $67{,}25 \pm 7$ years; and $66{,}5 \pm 6$ years, respectively. None of the patients had a history of inflammatory arthritis or crystal-induced arthritis. Human articular cartilage (leaf shape; size: length: 16 ± 5 mm and width: 8 ± 2 mm) was obtained from the femoral side, selecting the non-overload compartment: lateral condyle in varus deformity and medial condyle in valgus cases. Only cartilage that looked normal microscopically was used for this study. Samples collected at joint arthroplasty were transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 100 U/ml penicillin and 100 mg/ml streptomycin.

Isolation and dedifferentitation of human articular chondrocytes. Articular chondrocytes were isolated as previously described[12]. To induce chondrocyte dedifferentiation cells were harvesting by TrypLE (Invitrogen) and cultured on monolayer for 6–7 passages.

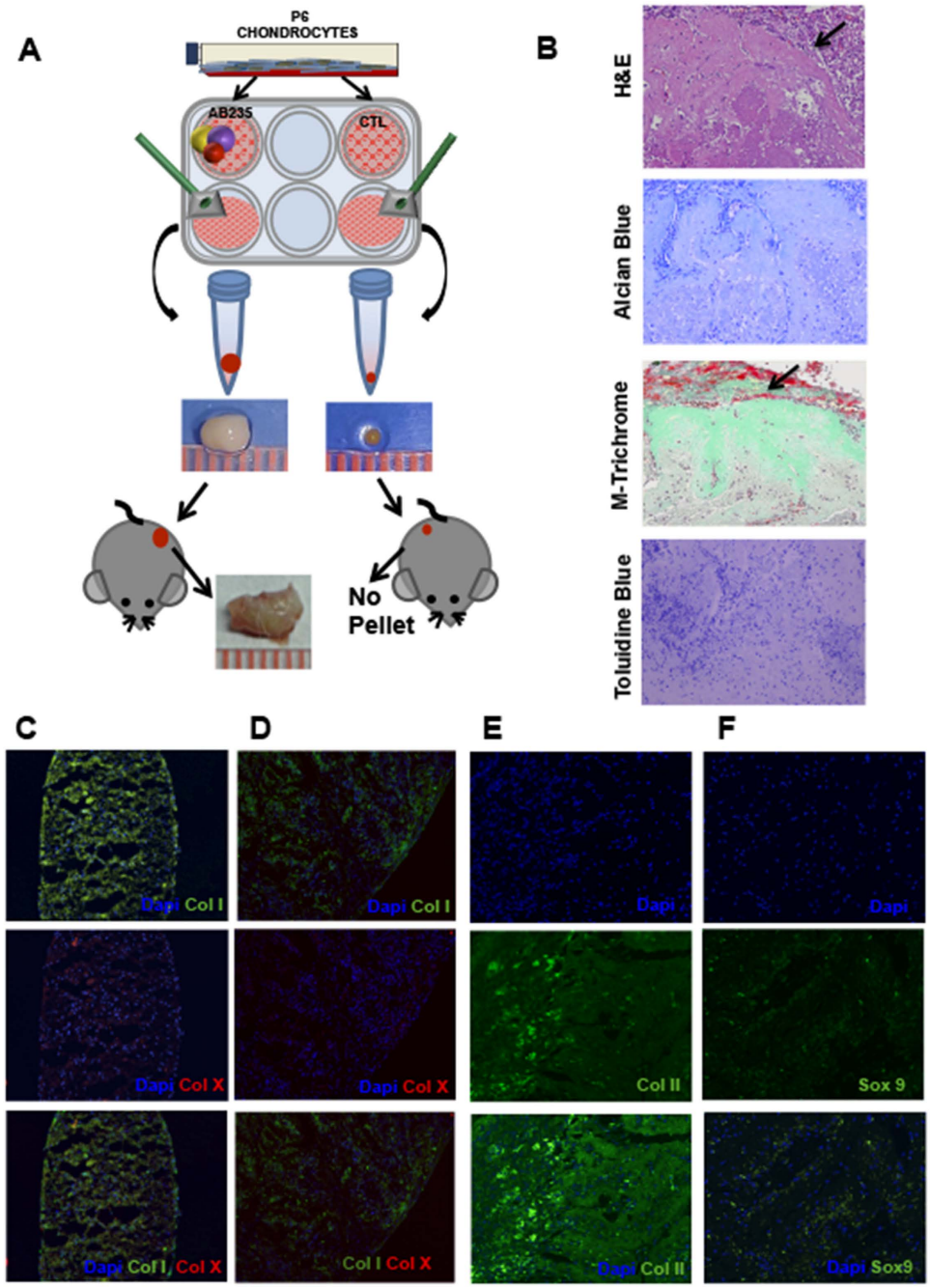

Figure 4. AB235 induces chondrocyte redifferentiation *in vivo*. (**A**) Schematic representation of the experimental design showing images of representative pellets before and after implantation into mice and integration of AB235-treated pellets with the surrounding tissue. (**B**) Sections of AB235-treated pellets harvested from mice and stained for H&E, Masson's Trichrome, Alcian Blue and Toluidine Blue show a robust staining for mature, cartilage-like ECM. Black arrows indicate the edge of the pellet in H&E and Masson's Trichrome stained sections while the edge of the pellet is not visible in Alcian Blue and Toluidine Blue stained sections. **(C–F)** Representative images of immunofluorescence analysis of cartilage markers. Stained sections of fibrotic marker type I collagen (Col I) and hypertrophic marker type X collagen (Col X) in both control pellet grown *in vitro* (**C**) and AB235-induced pellet harvested from mice (**D**). Expression of the chondrogenic markers Col II and Sox 9 in AB235 induced pellet sections after the *in vivo* assay (**E**,**F**). Original magnification 10× for (**C**,**D**); 20× for (**E**,**F**).

Chondrogenic differentiation in cell pellet culture. Chondrocytes were maintained at 37 °C in a humidified atmosphere containing 20% O2 and 5% CO2 usin a protocol detailed in Supplementary data. Briefly, 200,000 cells/ml were grown using a pellet system as described before[11]. Control pellets

were grown in DMEM–high glucose (Sigma) Supplemented with 10% foetal bovine serum (FBS, Gibco, composition is listed in supplementary data), 50 μg/μL of l-ascorbic acid 2-phosphate (Sigma), 1% penicillin-streptomycin (Sigma) and 1% ITS (Insulin-Transferrin-Selenium, Gibco). To induce redifferentiation, 10 ng/ml of AB235 was added fresh during each media exchange every 48 hours. The AB235 chimeric ligand was generated as previously described[10] and was dissolved in a solution (1:20 v/v) of 10 mM sodium acetate buffer (pH 5.0) and PBS containing 0,1% bovine serum albumin (BSA).

RNA isolation and real time-PCR analysis. Total cellular RNA isolation, cDNA generation and Real-time PCR were performed as described in Supplementary data. Primer sequences used in this study are summarized in Table S1 (Supplementary data).

Histological and immunohistochemical analysis. Described in detail in Supplementary data.

***In vivo* assay.** *In vivo* experiments were performed in immunodeficient NOD SCID (NOD.CB17-Prkdc^{scid}/NcrCrl) mice purchased from Charles River (Barcelona, Spain). Cell pellets obtained after 6 weeks of chondrogenic induction were transplanted into the back subcutaneous tissue of mice anesthetized (n = 6) by isoflurane inhalation (described in detail in Supplementary data). *In vivo* assays were carried out in accordance with the approved guidelines of University of Granada following institutional and international standards for animal welfare and experimental procedure. All experimental protocols were approved by the Research Ethics Committee of the University of Granada.

Statistical analysis. All graphed data represent the mean + /-SD from at least three experiments. Differences between treatments were tested using the two tailed Student´s T test. Assumptions of Student´s T test (homocedasticity and normality) were tested and assured by using transformed data sets [log(dependent variable value + 1)] when necessary. P-values < 0.01 (**) were considered statistically significant in all cases.

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Author Contributions

G.J. and E.L.-R. design study, data acquisition, data analysis and interpretation, drafting the article. W.K., J.C. I.-B., S.C. and P.C.G. data interpretation, provision of material, drafting the article, final approval of submitted manuscript. E.M.: provision of patients, drafting the article. F.A. and E.C. provision of material, data analysis. M.P. conception and design of the study, data analysis and interpretation, drafting the article. J.A.M. conception and design of the study, data analysis and interpretation, obtaining of funding, drafting the article. All authors: final approval of submitted manuscript.

Additional Information

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