Translational research for nasal septum cartilage regeneration with chondrocytes derived from differentiated human adipose mesenchymal stem cells

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ABSTRACT: Mesenchymal stem cells (MSCs) are multipotent cells isolated from various tissues, mainly from the bone marrow and adipose tissue. Their ability to differentiate into osteoblasts, chondrocytes or adipocytes renders them a promising clinical tool for injury repair and tissue regeneration. In the current study, MSCs were isolated from human adipose tissue (hAD-MSCs) and were triggered to differentiate into chondrocytes *in vitro*. Expression of mesenchymal stem cell markers, such as CD90 and CD73, in combination with the absence of hematopoietic markers, such as CD45, proves via flow cytometry the successful isolation of MSCs. Histologic staining with Toluidine blue and real time PCR analysis for the expression of the chondrogenic marker aggrecan (*ACAN*) verified the successful chondrogenic differentiation of AD-MSCs. Using Poly Lactic-Acid as scaffolding material, a three-dimensional scaffold with customized architecture, controlled porosity and interconnected porous structure was fabricated using 3D printing. The produced scaffold represents the morphology of the nasal septum cartilage. We aspire, to see this scaffold with the differentiated chondrocytes and culture the complex under the appropriate micoenvironmental conditions of a bioreactor system in order to regenerate a potential cartilage transplant. This *in vitro* study expands the potentials of human AD-MSCs to be used in clinic for alleviation of cartilage defects and tissue engineering in Greece and worldwide.

Key Words: Mesenchymal Stem Cells, Chondrocytes, Nasal Septum, Cartilage, 3D Printing, Flow Cytometry,

INTRODUCTION

MSCs are non-immortal multipotent cells, able to form fibroblast-like colonies[1]. MSCs can differentiate into osteoblasts, chondrocytes and adipocytes[2], but also into muscle cells, tenocytes, skeletal myocytes, neurons and endothelial cells under the appropriate *in vitro* inductive conditions[3-5] .

Human MSCs are mainly isolated from bone marrow and adipose tissue, but also from amniotic fluid, synovial tissue, skeletal and cardiac muscle, periosteum and foetal tissues[6-8]. After isolation MSCs must be cultured under sterile and strictly controlled conditions in minimal medium supplemented with serum in tissue culture plastic. After an initial delay, they start to proliferate rapidly, depending on the tissue source, the age, and the condition of the donor.

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MSCs express a number of surface markers, even though none of these is specific. They do not express any hematopoietic markers, such as CD14, CD34 and CD45. However, they express CD44, CD29, CD73, CD90, CD166, CD105 and others[6]. MSCs are non-immunogenic as they do not express MHCII, CD40 or CD80 (Table 1)[10].

In vivo studies have proven that injected MSCs have the ability to engraft into healthy and injured tissues with promising results for injury repair[7]. Their beneficial effects rely either on their differentiation potential into several cell types or on their ability to locally promote tissue regeneration via an autocrine, paracrine or juxtacrine action, without initiating an immune respons[11,12]. Systemic delivery of MSCs has also been reported by several groups, demonstrating the trafficking, adhesion and engraftment of MSCs to any site of injury[13].

MSCs are capable to differentiate into chondrocytes in 3D culture systems in the presence of dexamethasone, ascorbate and TGF-β[14]. Histological analysis of chondrocytes shows positive staining with toluidine blue[15]. Tissue engineered transplant describes the process of reconstituting mammalian tissue, both structurally and functionally. Such a process can be achieved either entirely *in vitro* or partially *in vitro* and then completed *in vivo*[16]. The process of tissue engineering often begins with a scaffold which is a three-dimensional structure. Cells are embedded in the matrices or penetrating the scaffold and promote tissue regeneration. There are many conventional techniques for scaffold fabrication suffering from several limitations, such as precise pore size, pore geometry and high mechanical strength.

Three-dimension printing can fabricate almost any object geometry using a layer-by-layer building method. It works as an ordinary 2D inkjet printer (length, width) using height as the third dimension. Furthermore, instead of ink as a material, it uses a liquid binder solution that is deposited on a power bed adding layers of the material to the designed structure. The methodology used to achieve 3D printed scaffolds starts with CAD (Computer Aided Design) that must be drawn or taken from known organ structures. A different approach is by taking cross-sectional slices and images from a computer tomography (CT) where they can be compiled into a 3D image. Finally, the designed geometry of the object can be produced with CAM (Computer Aided Manufacturing).

Essential details regarding the optimal conditions, tissue source and the matrix material, as well as clinical safety and efficacy remain to be tested. The raw data obtained from X-ray tomography and Magnetic Resonance Imaging (MRI) produce a 3D image demonstrating a cartilage part, which could reconstruct a cartilage defect. This image can then be 3D printed using a vast variety of material combinations and techniques to produce a porous matrix, where chondrocytes can colonize. The whole process can take place in a bioreactor.

Table 1. Cell surface markers expressed by MSCs. Adapted by Zimmerlin et al., Haynesworth et al., and Le Blanck at al.[17-19]

Cell surface markers expressed on mesenchymal stem cells.	
Positive	MHC class 1, HLA-ABC, CD13, CD29, CD34, CD44, CD71, CD73, CD90, CD105, CD106, CD166, Stro-1, VCAM-1, ALCAM-1, ICAM-1
Negative	MHC class II, HLA-DR, HLA-DP, CD11, CD14, CD18, CD31, CD34, CD38, CD40, CD45, CD80, CD86, CD106

Materials and Methods

Cell Isolation and expansion

Adipose tissues were collected after informed consent of all donors and in accordance with the institutional bioethics committee. Human adipose derived mesenchymal stem cells (AD-MSCs) were isolated from adipose tissue by enzymatic dissociation. In particular, the tissue sample was extensively washed with PBS (GIBCO, Thermo Fisher Scientific, Waltham, MA USA) containing penicillin/streptomycin (P/S) (Sigma-Aldrich, St. Louis, MO, USA) in order to remove debris. The tissue was placed in a sterile petri dish with 4mg/ml collagenase type I and 2mg/ml dispase type II (Roche, Basel, Switzerland) prepared in α-MEM medium (GIBCO, Thermo Fisher Scientific, Waltham, MA USA) with P/S and using sterile surgical blades (Paragon, UK) tissue was minced into small segments. The sample was digested for 1h at 37°C and in order to facilitate further the digestion the sample was pipetted up and down with a 10 or 25ml pipette several times intermittently. Single cell suspension was obtained by passing the cells through a 70 μm cell strainer, avoiding the solid aggregates. AD-MSCs were obtained by centrifugation at 1000 rpm for 5min. The supernatant was removed and the pellet

was resuspended with MSC medium (a-MEM, 15% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA USA), 2mM Glutamine, 0.1mM L-ascorbic acid phosphate, 100U/ml penicillin, 100mg/ml streptomycin). The primary cells were cultured in T75 flask (Corning, Sigma-Aldrich, St. Louis, MO, USA) at 37 C and 5% CO_2 for about 2 weeks until they reached confluency and were defined as passage 0. The cells, that had a typical fibroblast-like spindle shape, were passaged at a ratio of 1:3. The adherent AD-MSCs were expanded and either assessed for gene expression and cell surface marker expression or induced to differentiate towards chondrogenic, adipogenic and osteogenic lineages. The cells used in subsequent experiments were between passages 3 and 6.

2. CHARACTERIZATION OF UNDIFFERENTIATED AD-MSCS

Flow cytometry was performed to identify the expression of specific cell surface antigens that are used as markers for immunophenotypic characterisation of AD-MSCs.

The cells were harvested when reaching 90-100% confluency. Sufficient volume of trypsin-EDTA was added and the cells were placed in the incubator for 5 min. After verifying under the microscope that more than 90% of the attached cells have become detached from the surface, MSC medium was added in order to allow the serum contained in the medium to neutralize the trypsin reaction. The cells were centrifuged at 1000 rpm for 5min, were resuspended in MSC medium and were counted using trypan blue to determine viability. Because there is no single marker to identify AD-MSCs, a multicolor identification with a combination of fluorochrome-labeled antibodies for different surface antigens was used. For each analysis of surface epitope characterization cells were rinsed in PBS and resuspended at a density of 0.3-0.5 x 10^6 cells/100μl FACS buffer (0.1% sodium azide, 1% BSA in PBS). Additionally, a sample containing untreated cell suspension as a control for autofluorescence was prepared. The cells were first blocked with 5μg of human IgG (Sigma-Aldrich, St. Louis, MO, USA) for 15min on ice and subsequently stained with the following fluorochrome conjugated antibodies (all obtained from Biolegend, San Diego, CA, USA): CD90-FITC CD73-PE-Cy7 (both expressed by AD-MSC) and CD45-APC (negative marker). For each sample the 7-Aminoactinomycin D (7-AAD) Viability Staining Solution was also included in order to define a region for live cells (dead cell exclusion). 7-AAD is a fluorescent chemical compound with a strong affinity for DNA, which can only pass through disrupted cell membranes. Thus 7-AAD stains dead cells, while living cells with intact cell membranes will remain unstained[20]. Cells were incubated with the above mentioned combinations of antibodies for 20 min in the dark on ice. Stained cells were washed twice with FACS buffer (PBS, 1% BSA) and analyzed. Guava easyCyte flow cytometer (Merck-Millipore, Darmstadt, Germany) with the pre-installed software for data acquisition and analysis was used. In addition, quality control was performed by including the analysis of fluorescent beads to validate the function of the lasers before each analysis. A total of 10,000 events were acquired for each sample. Unstained cells were used as a control to set the gates and analysis region.

3. CELL DIFFERENTIATION ASSAYS

A hallmark of MSCs is their multipotency, which means that by using specific inductive culture media it is possible to direct MSC differentiation to specific cell types *in vitro*. However, not all MSCs in cultures present this ability due to cellular senescence or lack of optimal cocktail that drives differentiation. Based on these observations a trilineage differentiation assay of the AD-MSCs was performed in order to confirm their multipotency.

a. Chondrogenic differentiation

AD-MSCs were seeded in 6-well plates until confluency and were differentiated into chondrocytes by culturing the cells into chondrogenic differentiation medium consisting of a-MEM supplemented with 15% FBS, 2mM Glutamine, 0.1mM L-ascorbic acid phosphate, 100U/ml penicillin, 100mg/ml streptomycin, ITS, 100nM dexamethasone and 10ng/mL transforming growth factor-β1. Cells were cultured for 7, 14 and 21 days with the differentiation medium (medium changes twice per week). Evaluation of the chondrogenic differentiation was carried out by staining with Toluidine blue (Sigma-Aldrich, St. Louis, MO, USA). Cells were fixed in 10% formalin for 30min, washed 2 times with PBS and stained with 0.1% Toluidine blue for 30min in order to highlight the presence of proteoglycans and subsequently the level of chondrogenic differentiation. Morphological changes could be observed by comparing uninduced and induced AD-MSCs. Gene expression analysis was also performed as described below.

b. Osteogenic differentiation

The osteogenic differentiation capacity of AD-MSC was investigated in cultures in the presence of osteogenic medium, a-MEM supplemented with 15% FBS, 2mM Glutamine, 0.1mM L-ascorbic acid phosphate, 100U/ml penicillin, 100mg/ml streptomycin, 10nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 1.8 mM KH_2PO_4 and 5mM glycerolphosphate (Sigma-Aldrich, St. Louis, MO, USA). After culture for 7, 14 and 21 days with the differentiation medium (medium changes twice per week) calcium accumulations were observed under the microscope.

c. Adipogenic differentiation

The adipogenic differentiation capacity of AD-MSC was investigated in the presence of adipogenic medium, a-MEM supplemented with 15% FBS, 2mM Glutamine, 0.1mM L-ascorbic acid phosphate, 100U/ml penicillin, 100mg/ml streptomycin, 0.5μM dexamethasone, 0.5mM IBMX (Sigma-Aldrich, St. Louis, MO, USA), 10μg/ml insulin and 60 μM indomethacin (Sigma-Aldrich, St. Louis, MO, USA). After culture for 7, 14 and 21 days with the differentiation medium (medium changes twice per week) lipids droplets were observed under the microscope.

4. MRNA ISOLATION AND REAL-TIME RT-PCR ASSAYS

Total mRNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol and Epoch microplate spectrophotometer was used to quantify RNA. cDNA synthesis was performed by using 0.5μg RNA as template and the PrimeScript RT reagent kit (Takara Shuzo Co., Kyoto, Japan). Real-Time PCR was carried out in duplicate using KAPA SYBR Fast® Mix (KK4601 Kapa Biosystems, Woburn, MA, USA) in StepOne™ (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA). The PCR specifications were as follows: 95˚C for 10 minutes, 40 cycles for 5 seconds at 95˚C, 60˚C for 30 seconds. Beta-2 microglobulin (B2M) was used as reference gene and aggrecan expression was used to confirm chondrogenic differentiation.

5. SCAFFOLD FABRICATION AND CAD - CAM/ 3D PRINTING

The integration of an advanced manufacturing technique with a biocompatible biomaterial of controlled porous architecture was investigated in this study aiming to deliver a personalized septal cartilage scaffold. Based on the Poly Lactic-Acid material PLA, a three-dimensional scaffold with customized architecture, controlled porosity and totally interconnected porous structure was successfully fabricated using Fused Deposition Modeling (FDM) method, one of the 3D printing techniques.

For the experiment, firstly one porous scaffold was designed using SolidWorks as a CAD software, and afterwards the design was converted to an STL format (STL being derived from the name Stereolithography). The STL file was then loaded to Slic3r software for setting up the fabrication parameters. The scaffold sliced into 9 layers and the infill parameters were set to, fill density: 22%, fill pattern: honeycomb and the top/bottom fill pattern: rectilinear. The temperatures of the liquefier and the heated bed were set to 210ºC and 50ºC respectively. After settings, G-Code was created and the septal cartilage was fabricated with PRUSA I3, a 3D Printing machine.

RESULTS

Characterization of AD-MSCs

After cellular adhesion, the cells displayed the typical fibroblast-like spindle shape of MSCs (Fig 1).

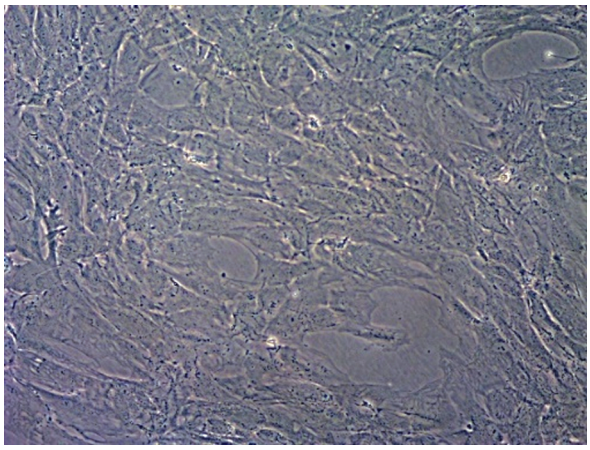

Figure 1. Morphology of AD-MSC culture *in vitro*.

The cultured AD-MSCs expressed the cell surface markers CD90 and CD73 and were negative to CD45, as it was expected based on the literature (Fig 2). The absence of stained cells with 7-AAD viability staining solution, confirmed the high viability of the evaluated cell population.

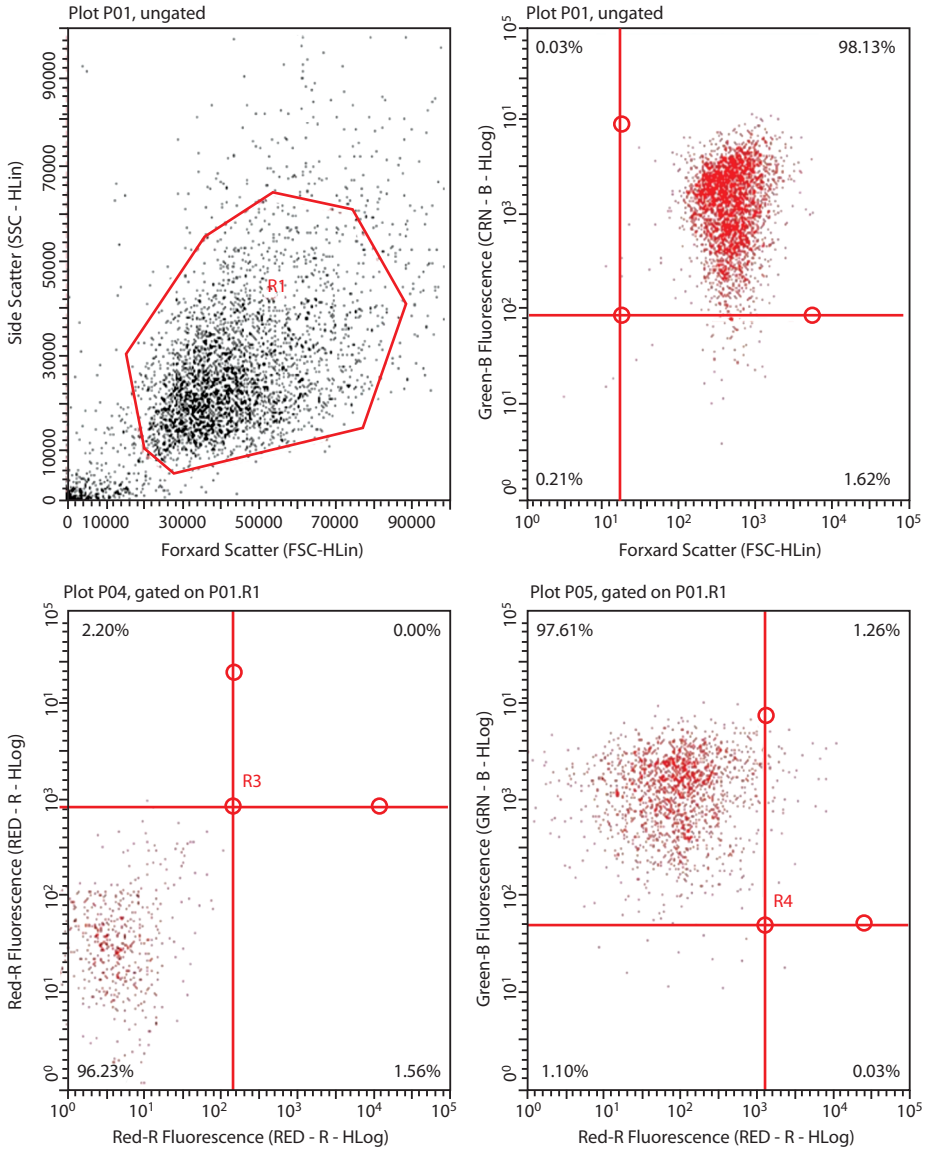

Figure 2. Characterization of AD-MSCs by flow cytometry. **a)** Gating **b)** Representative diagram showing the expression of positive mesenchymal markers y-axis CD90 FITC, x-axis CD73 PE-Cy7, **c)** Representative diagram showing the expression of the negative mesenchymal marker y-axis CD45 APC, and x-axis 7AAD Viability Staining Solution **d)** Representative diagram showing the expression of the negative mesenchymal marker x-axis CD45-APC, and the positive marker y-axis CD90-FTC.

Chondrogenic differentiation

After 7, 14 and 21 days of culturing in differentiation media the AD-MSC were positively stained with toluidine blue. Toluidine blue is a majorly used thiazine metachromatic dye with high affinity for acidic tissue components, thereby staining tissues rich in DNA and RNA. When bound to cartilage, toluidine blue becomes from blue to red (depending on the Chondroitin sulfate content)[21]. The expected colour change of the stain was observed from day 7 to day 21 as the stem cells were differentiated into chondrocytes (Fig 3).

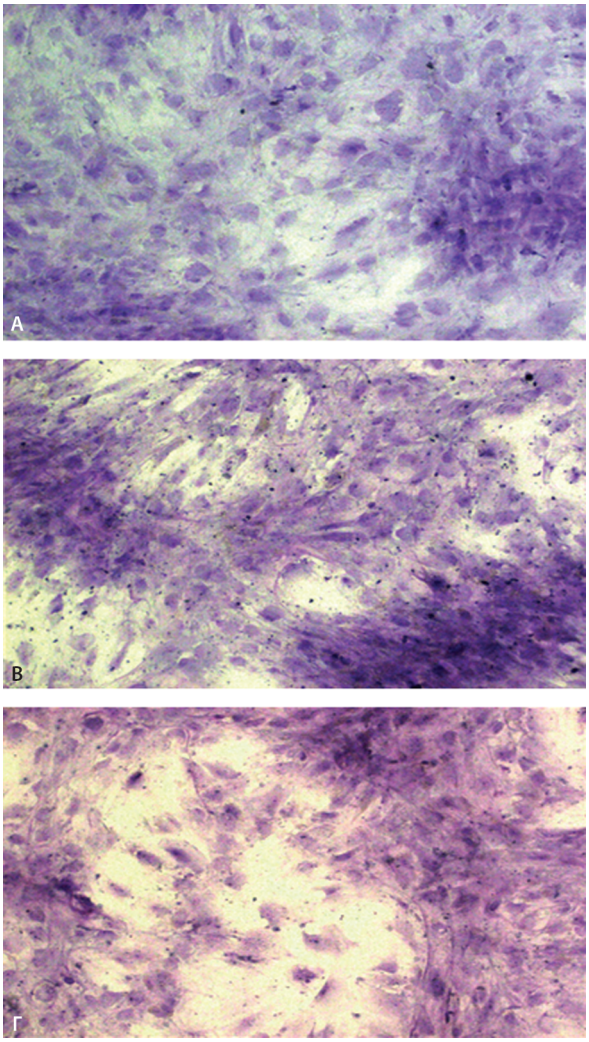

Figure 3. *In vitro* chondrogenic differentiation. AD-MSC stained with the metachromatic stain toluidine blue for up to 21 days.

For further characterisation of the chondrocytes, at the mRNA level this time, Real-time PCR was performed in total RNA at days 7, 14 and 21 after induction of differentiation. The mRNA of the gene *ACAN* gene, which is expressed in cartilage, was increasing during chondrogenic differentiation (Fig 4).

Three-dimensional septal cartilage design and fabrication

An FDM 3D printing machine (PRUSA I3) and PLA (Poly Lactic-Acid) as a material was used to fabricate a septal cartilage scaffold to investigate cell biocompatibility and mechanical properties into a bioreactor with different principles of bioprocesses.

For the experiment, one porous scaffold was designed using SolidWorks as CAD software and the dimensions were based on the basic anatomy of a septal cartilage (Figure 5 and 6).

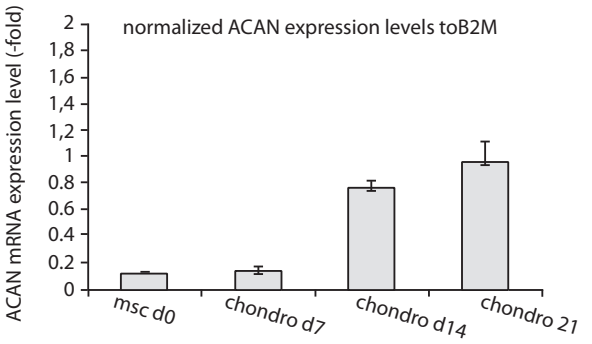

Figure 4: Real-time PCR analysis of the chondrogenic expressed gene *ACAN* geneat at 7, 14 and 21 days post-induction. Results are normalized to the expression of the housekeeping gene B2M

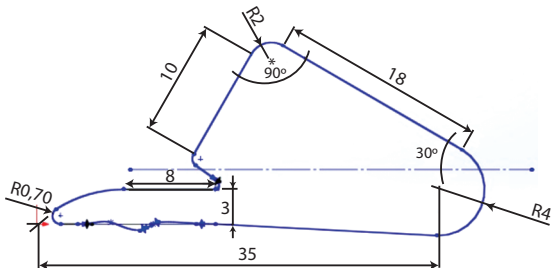

Figure 5. Schematic of the computer aided design model in 2 dimensional view.

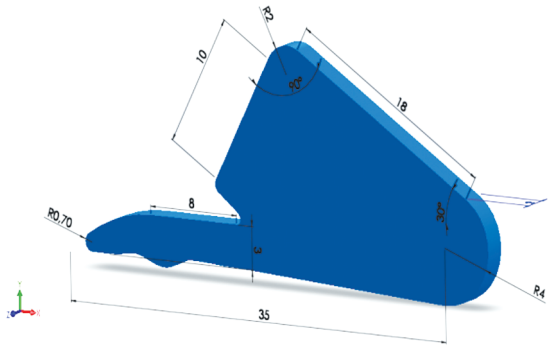

Figure 6. Schematic of the computer aided design septal cartilage in 3 dimensional view.

The design was exported into an STL format (STL being derived from the name stereolithography) and transferred to the Slic3r software to export the G-code for 3D Printing. The infill settings that were used for the pores of the scaffold are:

Fill density: 22%
Fill pattern: Honeycomb
Top/Bottom Fill pattern: rectilinear
(Figures 7 and 8).

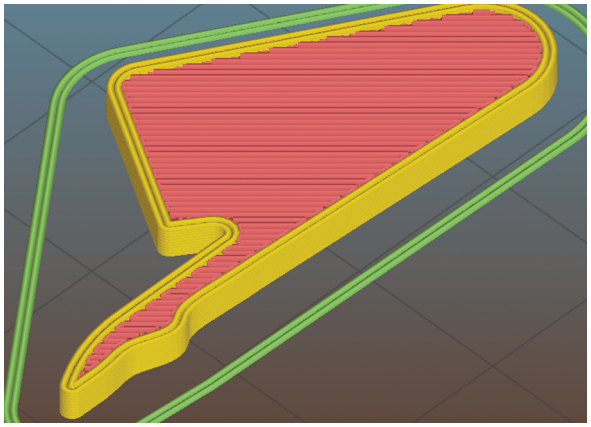

Figure 7. Schematic of the porosity of the septal cartilage, rectilinear.

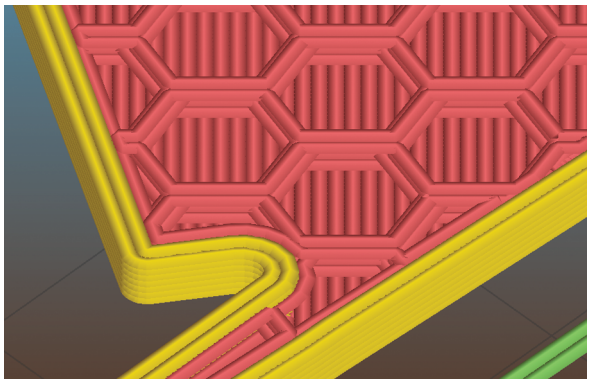

Figure 8. Schematic of the porosity of septal cartilage, honeycomb.

The scaffold model was sliced into 9 layers and it was fabricated using the G-Code exported from Silc3R (Fig. 9). The liquefier temperature was set at 210^0 C ± 2^0 C and the platform temperature was set to 50^0 C. After the fabrication, the scaffold was removed from the base and stored prior to usage.

DISCUSSION

Human adipose-derived MSCs are being isolated, cultured in vitro and chondrogenic differentiation is induced. The process of differentiation into cartilage is monitored in different levels. The expression of chondrocytic surface markers in protein level is proven via flow cytometry and staining. While, the expression

Figure 9: Polymer scaffold of septal cartilage.

of other genes representative of chondrocytes, such as ACAN (aggreecan), is tested in mRNA level using qPCR, showing the expected pattern of increasing expression.

In parallel, a three dimensional scaffold representing precisely the morphology and size of a nasal septal cartilage was produced. CAD/CAM technology was used to design of the scaffold which was subsequently manufactured using 3D printing. The material used was Poly Lactic-Acid (PLA), carefully chosen as a "study" material for the purpose of translational research.

The next step in this study will be the in vitro co-culture of the 3D chitosan/gelatine scaffold (still under development) with the differentiated chondrocytes in a perfusion bioreactor. During this process the cells are expected to be embedded in the matrix and shape a potential cartilage transplant. In the near future, scaffolds representing cartilage features of other body parts with a high rate of defects, such as knee cartilage, will be designed and produced. The same technology of 3D printing can be employed with other combinations of materials, in pursuit of better mechanical properties, biodegradability, and biocompatibility of the transplants. Personalized cartilage scaffolds for clinical use it is also possible and anticipated to be more and more available as the methods are being perfected.

Our first limitation of cartilage transplant production is the chamber size of the bioreactor. In our commercialised bioreactor (BOSE/TA instruments) only small cartilage parts, size ranging to 2.5X2.5.bX8.0cm

can be cultured, restricting the options. To overcome this limitation we are aiming to design and produce via 3D printing new bigger chambers, appropriate to host scaffolds of any possible size and morphology.

This state-of-the-art technology has being made possible after the implementation of the project REMEDIC (www.remedicproject.eu), which provided for the School of Medicine of the Aristotle University a complete functional unit of Regenerative Medicine. The programme has also provide funds for translational research that is now being carried out aiming to knowledge dissemination new skills development and keeping our Scholl updated and competitive with respect to Regenerative Medicine research.

Μεταφραστική έρευνα για την αναγέννηση ρινικού διαφράγματος με χονδροκύτταρα προερχόμενα από διαφοροποιημένα μεσεγυματικά βλαστικά κύτταρα λιπώδους ιστού ανθρώπου

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Περίληψη: Η εργασία αφορά στη μεταφραστική έρευνα ιστοτεχνολογίας και συγκεκριμένα στη δημιουργία ανθρώπινου ρινικού διαφράγματος με τη χρήση ηλεκτρονικά υποβοηθούμενου σχεδιασμού και τρισδιάστατης εκτύπωσης τρισδιάστατου (3D) πορώδους ικριώματος χιτοζάνης/ζελατίνης (CAD/CAM). Το ικρίωμα θα χρησιμοποιηθεί για να αποικιστεί από χονδροκύτταρα που προκύπτουν από διαφοροποιημένα μεσεγχυματικά κύτταρα ανθρώπου προερχόμενα από λιπώδη ιστό (Adipose Tissue Mesenchymal Stem Cells-AD- MSCs). Η όλη διαδικασία επιτυγχάνεται με τη χρήση βιοαντιδραστήρα. Τα μεσεγχυματικά κύτταρα είναι πολυδύναμα βλαστοκύτταρα που μπορούν να απομονωθούν από το μυελό των οστών και το λιπώδη ιστό. Τα κύτταρα αυτά έχουν τη δυνατότητα να διαφοροποιούνται, υπό εργαστηριακές συνθήκες, σε οστεοκύτταρα, χονδροκύτταρα, και λιποκύτταρα. Στην παρούσα μελέτη ανθρώπινα μεσεγχυματικά κύτταρα απομονώθηκαν από λιπώδη ιστό και καλλιεργήθηκαν in vitro. Η έκφραση των αντιγόνων επιφανείας CD90, CD73, σε συνδυασμό με την απουσία του μάρτυρα CD45 επιβεβαιώνουν την επιτυχή απομόνωση μεσεγχυματικών βλαστικών κυττάρων, με χρήση κυτταρομετρίας ροής. ΄Επειτα από 21 ημέρες από την επαγωγή στοχευόμενης διαφοροποίησης τα βλαστοκύτταρα διαφοροποιήθηκαν σε χονδροκύτταρα και χαρακτηρίστηκαν ιστολογικά με χρώση κυανού της τολουιδίνης και μοριακά με RT-PCR για δείκτες διαφοροποίησης όπως η αγκρεκάνη. Με τη χρήση του τρισδιάστατου εκτυπωτή δημιουργήθηκε υπό κλίμακα ικρίωμα ρινικού χόνδρου από PLA. Η διαδικασία θα ολοκληρωθεί με την εκτύπωση του υπό διερεύνηση υλικού χιτοζάνης/ζελατίνης σε 3D ικρίωμα και αφού εμποτιστεί με χονδροκύτταρα θα μεταφερθεί στον βιοαντιδραστήρα.

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