



## A study of crystalline biomaterials for articular cartilage bioengineering

Talia Gross-Aviv <sup>a,\*</sup>, Bryan B. DiCarlo <sup>b,1</sup>, Margaret M. French <sup>b,1</sup>, Kyriacos A. Athanasiou <sup>b,2</sup>, Razi Vago <sup>a,3</sup>

<sup>a</sup> Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer Sheva, 84105 Israel

<sup>b</sup> Department of Bioengineering, Rice University, Houston, TX 77003, United States

### ARTICLE INFO

#### Article history:

Received 22 November 2007

Accepted 16 March 2008

Available online 27 March 2008

#### Keywords:

Tissue engineering

Chondrocytes

Mesenchymal stem cells (MSCs)

Biomaterials

Coral

Hydrozoa

### ABSTRACT

This study examines the suitability of marine origin coral species, *Porites lutea* (POR) and the hydrozoan *Millepora dichotoma* (MIL), for use as novel three dimensional growth matrices in the field of articular cartilage tissue engineering. Therefore, mesenchymal stem cells (MSCs) and chondrocytes were grown on the skeletal material obtained from each of these two organisms to investigate their potential use as three dimensional scaffolding for cartilage tissue growth. Chondrogenic induction of MSCs was achieved by addition of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and insulin growth factor-I (IGF-I). Cell adherence, proliferation, differentiation and tissue development were investigated through six weeks of culture. Cartilage tissue growth and chondrocytic phenotype maintenance of each cell type were examined by cell morphology, histochemical analyses, expression of collagen type II and quantitative measures of glycosaminoglycan (GAG) content. The MSCs and the chondrocytes were shown good adherence to the scaffolds and maintenance of the chondrocytic phenotype in the initial stages of culture. However after two weeks of culture on MIL and three weeks on POR these cultures began to exhibit signs of further differentiation and phenotypic loss. The shown results indicated that POR was a better substrate for chondrocytes phenotype maintenance than MIL. We believe that surface modification of POR combined with mechanical stimuli will provide a suitable environment for chondrogenic phenotype maintenance. Further investigation of POR and other novel coralline biomaterials is indicated and warranted in the field of cartilage tissue engineering applications.

© 2008 Elsevier B.V. All rights reserved.

### 1. Introduction

Osteoarthritis is one of the main common diseases leading to the loss of articular cartilage [1,2]. One of the most challenging approaches in cartilage tissue repair is the *ex-vivo* bioengineering of tissue that will then be transplanted to the deficient or non-functioning cartilage zone. This approach comprises three main interactive components: a compatible cell source, a supportive biocompatible growth matrix and a supplement of bioactive molecules that promote cellular proliferation, differentiation and tissue development. Moreover, development of a suitable bioengineered implant will mimic native tissue structure and function after harmless biodegradation of the supportive matrix over time [1].

Cell source could be either fully differentiated cells of the desired tissue type or undifferentiated pluripotent cells, such as bone marrow derived mesenchymal stem cells (MSCs), which can be induced to

form the tissue of interest. The strategy of using differentiated cells is appealing because of the fact that these cells will naturally express the extra cellular matrix (ECM), collagen type II, glycosaminoglycans (GAG), cartilage oligomeric matrix protein (COMP), and other components of native cartilage tissues [3]. However, there are also some disadvantages in the use of fully differentiated chondrocytes in cartilage tissue engineering. These include the limited proliferative capacity of chondrocytes [1], and the difficulty in obtaining an adequate supply of cells needed for the high seeding densities required in cartilage tissue engineering [4]. In addition, chondrocytes tend to de-differentiate, exhibiting profound phenotypic changes, while growing in culture for long periods. The de-differentiated chondrocytes develop a fibroblastic phenotype while down regulating the production of collagen type II and up regulating the production of collagen type I, indicative in the loss of the chondrocytic phenotype [5]. Harvesting and culturing the large quantities of cells required in cartilage tissue engineering, while maintaining the chondrocytic phenotype, are indeed challenging tasks [6,7].

MSCs are progenitors that can differentiate into a variety of mesenchymal tissues such as: adipose tissue, muscle, skin or bone and cartilage. These cells proliferate extensively and differentiate into chondrocytes within short periods of time [8–11]. However, the engineered tissue can exhibit an unstable phenotype, expressing the phenotypes of several different tissues [8]. Chondrogenesis of MSCs

\* Corresponding author. Tel.: +972 8 6479087; fax: +972 8 6472983.

E-mail addresses: [taliag@bgu.ac.il](mailto:taliag@bgu.ac.il), [aviaviv177@gmail.com](mailto:aviaviv177@gmail.com) (T. Gross-Aviv), [bdicarlo@rice.edu](mailto:bdicarlo@rice.edu) (B.B. DiCarlo), [mmfrench@rice.edu](mailto:mmfrench@rice.edu) (M.M. French), [athanasiou@rice.edu](mailto:athanasiou@rice.edu) (K.A. Athanasiou), [rvago@bgu.ac.il](mailto:rvago@bgu.ac.il) (R. Vago).

<sup>1</sup> Tel.: +1 713 348 6393; fax: +1 713 348 5877.

<sup>2</sup> Tel.: +1 713 348 6385; fax: +1 713 348 5877.

<sup>3</sup> Tel.: +972 8 6477181; fax: +972 8 6472983.

can be induced by addition of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and insulin like growth factor-I (IGF-I) that may have synergistic effects enhancing the synthesis of chondrogenic products in the ECM, both *in-vitro* and *in-vivo* [12,13]. TGF- $\beta$ 1 acts at the early time of culture to induce chondrogenesis, while IGF-I enhances and maintains cell proliferation in culture [12].

A three dimensional (3D) scaffolding material, for use in tissue engineering, should provide the necessary structural, spatial and temporal environment necessary for cells to adhere, proliferate, organize and assemble complete tissues. This is of a particular importance in the field of cartilage tissue engineering due to the acellular, avascular and high impact environment in cartilage tissues [4,14,15]. Many types of materials have been suggested as candidate scaffolds for use in cartilage tissue engineering, such as: Synthetic polymers including the poly(glycolic acid) (PGA) [16,17] and poly(L-lactic acid) (PLLA) [18], which are easily produced and found to be biocompatible and biodegradable. However, cells have limited adherence to these matrices and the scaffolding material is mechanically unstable [14,15,19]. Other candidate scaffolds include natural materials such as collagen [20,21], alginates [22,23] and chitosan [24]. This group has low toxicity and a low inflammatory response. They are biodegradable and some of them (such as collagen) have been found to have the proper molecular cues to stimulate the expression of the chondrogenic phenotype [25]. However, most of them have poor mechanical integrity in comparison to native cartilage tissue as well as complex structures making manipulation difficult [14,19].

In the present study we examine the use of two marine origin biomaterials as novel scaffolding materials in cartilage tissue engineering. The first is the skeletal material of *Millepora dichotoma* (MIL), a sessile hydrozoan, typical to the shallow zones of tropical seas. The skeleton of MIL has proven to be an effective material for implant applications supporting chondral and subchondral remodeling [26]. The second is the coral skeleton of *Porites lutea* (POR), which has been extensively studied as a scaffold material for use in hard tissue engineering [27]. This coralline lattice possesses all the principal properties required of a bone graft substitute including: biocompatibility, biodegradability and transient mechanical strength. Moreover, it allows fast cellular invasion, adherence, proliferation [27–30] and an osteogenic capacity [28–31]. The skeletons of both of these organisms are made of a calcium carbonate ( $\text{CaCO}_3$ ) crystalline formation known as aragonite arranged in a lattice of pores measuring between 100 and 150  $\mu\text{m}$  [27,32]. The density of the two skeletons is: 2.5  $\text{g}/\text{cm}^3$  and 1.7  $\text{g}/\text{cm}^3$  for MIL and POR respectively [33].

The objective of this study is to examine the use of MIL and POR as scaffolding biomaterials for use in cartilage tissue engineering, comparing the capacity of these two biomaterials to provide a suitable environment for cartilage tissue development using MSCs or fully differentiated chondrocytes as cell sources, and compare the development of chondrogenic markers by MSCs and chondrocytes, on each of the coralline scaffolds.

## 2. Methods

### 2.1. Scaffold preparation

POR and MIL are both naturally occurring aragonite crystalline skeletal materials of common reef building organisms. In this study the naturally occurring POR were used, while MIL was cloned and fabricated at our lab according to procedure described by Abramovitch-Gottlib [34]. Colonies were cut into blocks and bleached with commercial hypochloric solution, rinsed with distilled water and dried in air. Samples were prepared by polishing to a thickness of approximately 0.5 mm and an area of 0.5  $\text{cm}^2$ , using an 8" grinder machine (model SPT 900). In order to remove inorganic residues the samples were rinsed in analytical grade  $\text{H}_2\text{O}_2$  solution (GERDROGEN 30% by weight; Riedel-de Haen). Organic residues were then removed

by a subsequent 2 N NaOH wash. Samples were then autoclaved (121 °C, 30 min) and dried overnight at 60 °C.

### 2.2. Cell culture

Chondrocytes, isolated from neonatal bovine knees were seeded on each scaffold and cultured in Dulbecco modified Eagle's medium (DMEM) (Biological industries, Israel) supplemented with 4.5 g/l D-glucose, 1.5 g/l sodium bicarbonate (Sigma), 1 mM sodium pyruvate (Biological industries, Israel), 10% (v/v) fetal calf serum (Biological industries, Israel), 1% L-Glutamine (Biological industries, Israel), 1% Pen-Strep-Nystatin Solution (Biological industries, Israel) and 50 mg/ml ascorbic acid (Sigma).

MSCs, (cell line ATCC/CRL 12424) passage 3 were seeded on the marine origin biomaterials. Cells were cultured using media (prepared as described above) supplemented with 10 ng/ml TGF- $\beta$ 1 (Cytolab, USA) during the first three days and 100 ng/ml IGF-I (MD biosciences, Israel) throughout the experimental period.

Cells were seeded at a concentration of 6400 cells per scaffold statically on the coralline biomaterials. The low cell seeding density was chosen due to high capacity of MSCs proliferation which rapidly infiltrates the matrices. A drop of media containing 6400 cells was placed on top of each biomaterials and allowed to remain in place by surface tension for 10 min before complete coverage in media. Cells were cultured at 37 °C in a humidified 10%  $\text{CO}_2$  incubator. Media replacements took place every two days. The progresses of the cultures were monitored daily by light inverted microscopy (Nikon, Eclipse TE300).

### 2.3. Scanning electron microscopy (SEM)

Stock solution of fixative was prepared using 0.2 M buffer phosphate, 10% paraformaldehyde (PFA) and 25% glutaraldehyde (GA) in distilled water (DW). Samples were rinsed twice with phosphate buffered saline (PBS) (Biological Industries, Israel) for 10 min and immersed in the fixative at 37 °C for 30 min. Then, samples were soaked in a serial gradient of ethanol: 50%, 75%, 90%, 95%, for 10 min each and three times in 100% ethanol for 10 min. The samples were washed and immersed in hexamethyldisilazane (HMDS) (Bel-Gar, Israel) and ethanol solutions in different volume ratios (1:2, 1:1 and 2:1, respectively). Solution residues evaporated over night in a chemical hood. Morphology of the samples was visualized using scanning electron microscopy (Quanta 200 ESEM/SEM, FEI).

### 2.4. Histochemical analyses

At each time point, samples were stained with safranin O for GAG content, von Kossa for bone nodules formation and oil red O for fat or lipids, according to procedures described below. The samples were photographed using a stereoscopic microscopy imaging system (Nikon, SMZ 1500) fitted with a digital camera (Nikon, DXM1200).

#### 2.4.1. Safranin O staining

A stock solution of 1% safranin O (Sigma-Aldrich; 0.5 g in 100 ml of isopropanol) was prepared. Samples were rinsed three times with PBS, fixed with 12% formaldehyde for 5 min and then stained with safranin O stock solution for 15 min. Serial ethanol gradients (50, 75, 90 and 95%, and twice 100%) were used to rinse staining residues from the sample.

#### 2.4.2. von Kossa staining

At the experiment day a fresh stock solution of 5% silver nitrate (Sigma-Aldrich) and 5% sodium thiosulfate (Sigma-Aldrich) were prepared in DW. Samples were rinsed with 150 mM NaCl three times, fixed in 95% ice cold ethanol, rinsed with DW and incubated under a UV light in the presence of silver nitrate stock solution for 30 min.

Following the incubation the samples were rinsed three times with DW and incubated in the presence of sodium thiosulfate stock solution for 2 min.

#### 2.4.3. Oil red O staining

A stock solution of oil red O (Sigma-Aldrich; 0.5 g in 100 ml of isopropanol) was prepared and passed through a filtering paper. 6 ml of the solution were mixed with 4 ml of DW, left for 1 h at room temperature, and filtered through a 0.2 µm prior to use. Samples were washed with PBS, fixed with 12% formaldehyde in PBS for 15 min, stained with 300 µl of oil red O stock solution for 1 h at room temperature and rinsed three times with DW.

#### 2.5. Immunofluorescence analyses

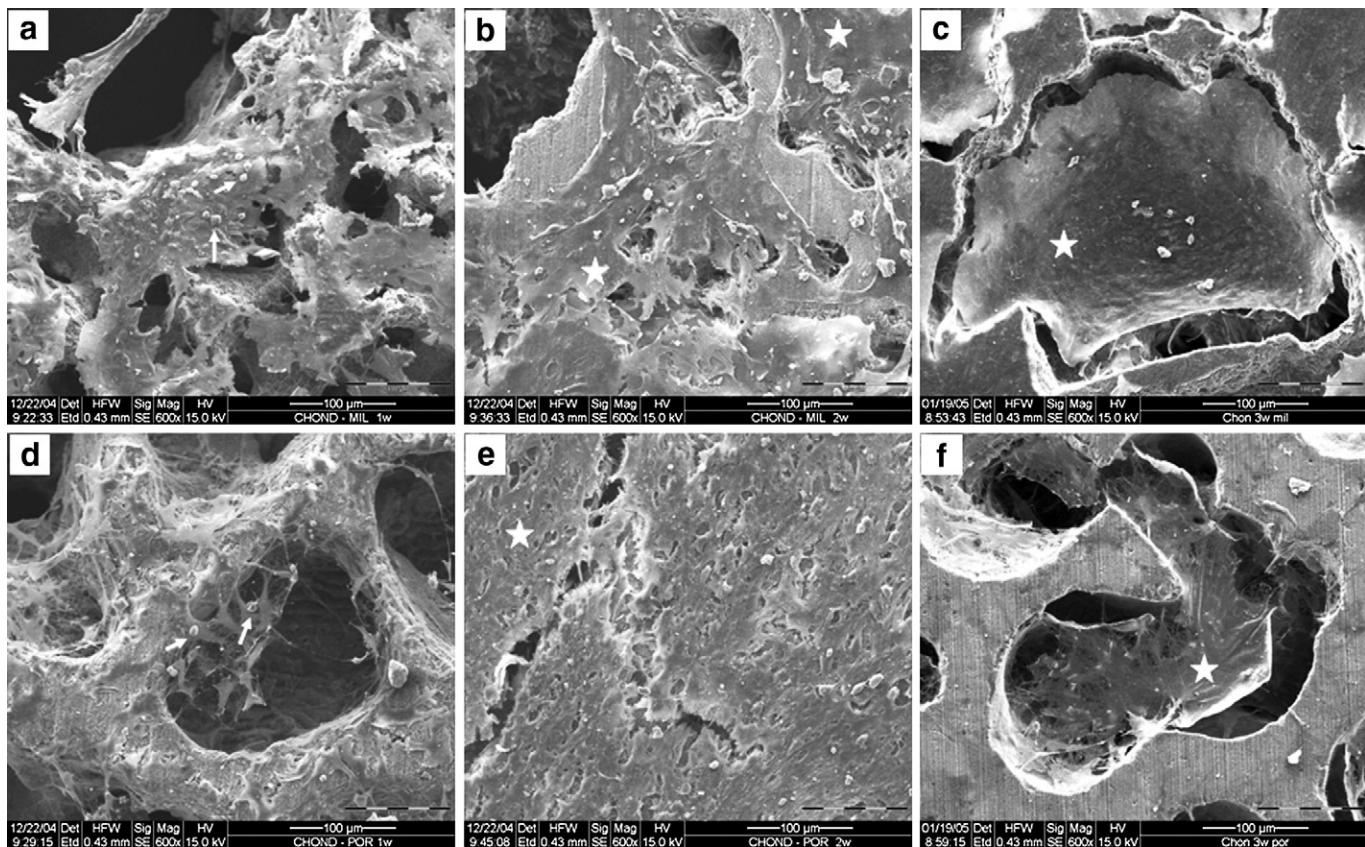
Samples were rinsed gently twice with PBS and fixed with cold 4% GA for 5 min at 4 °C. Fixed samples were rinsed 3 times with PBS (5 min per wash) and nonspecific antibody binding sites were blocked by incubating samples in blocking solution containing PBS and 0.5 g/100 ml bovine serum albumin (Biological industries, Israel) for 15 min. The samples were placed in a humid chamber and stained with goat polyclonal Immunoglobulin G (IgG) against type I collagen (Santa Cruz Biotechnology, USA) or goat polyclonal IgG against type II collagen (Santa Cruz Biotechnology, USA), diluted in a 1:50 ratio with PBS at 4 °C, over night. Three washes (5 min per wash) with PBS were performed and then the samples were covered with a 1:50 diluted solution of donkey anti-goat IgG fluorescein isothiocyanate (FITC) conjugated affinity purified antibody (Santa Cruz Biotechnology, USA) or rhodamine red-X-conjugated affiniPure donkey anti-goat IgG (H+L) (Jackson Immunoresearch Laboratories, INC., USA) in PBS for 60 min at room temperature. After addition of the secondary antibodies, samples were washed again 3 times with PBS and photographed using an

inverted fluorescence microscope (Nikon, Eclipse TE300) with FITC or rhodamine filters.

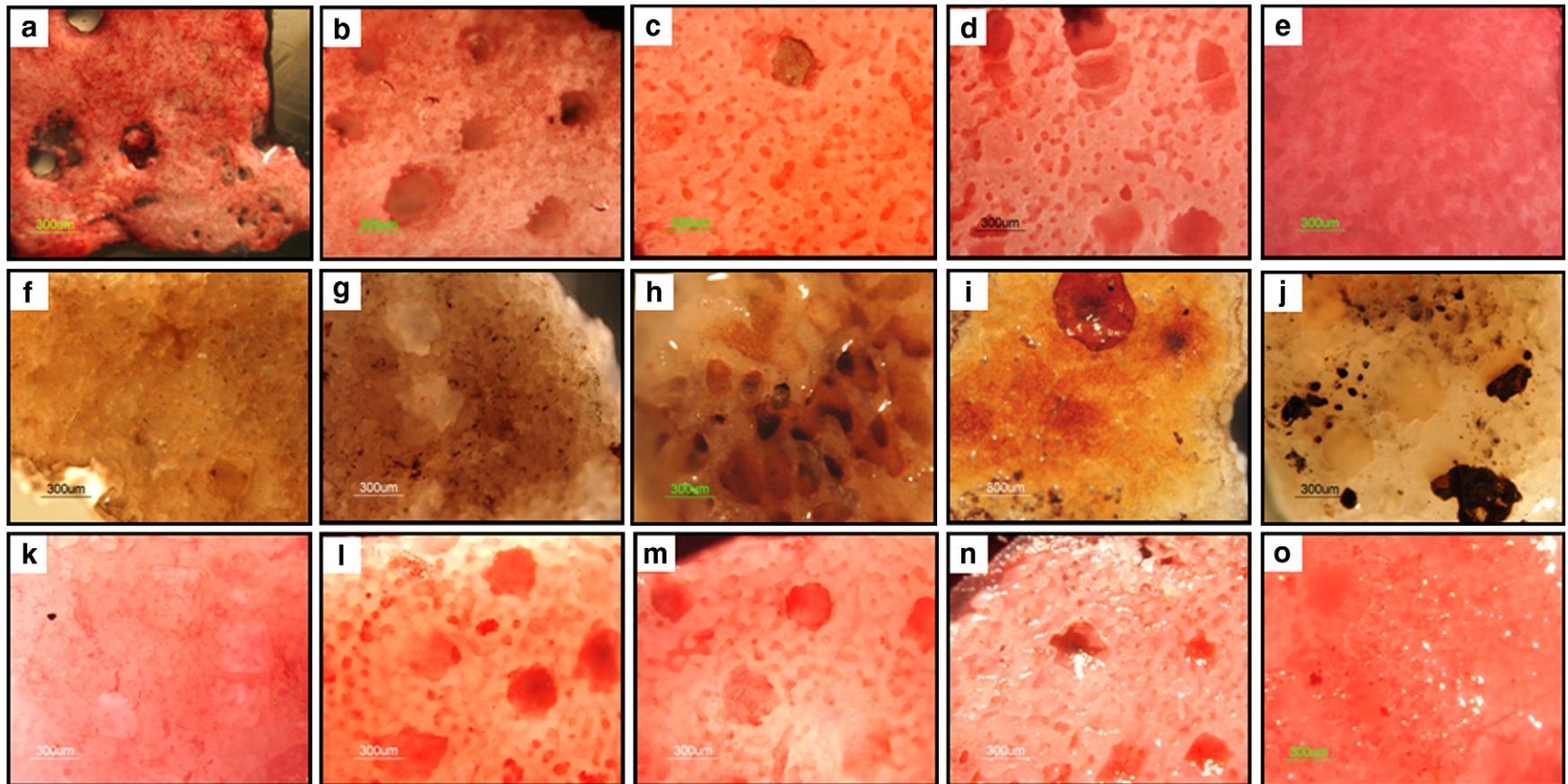
#### 2.6. Biochemical analyses

GAG content was analyzed using the blyscan sulfated glycosaminoglycan assay (Biocolor, Ireland). Seeded samples were transferred to 0.3 ml of solution containing 1 mM EDTA (Riedel-deHaen, USA), 20 mM sodium phosphate buffer (pH=6.8) (Biological industries, Israel), 2 mM dithiothreitol (Sigma) and 300 µg/ml Papain (Sigma). The samples and blyscan sulfated glycosaminoglycan standards were incubated in the solution over night at 60 °C. One ml of blyscan dye reagent was added to 50 µl aliquot of digested sample, mixed and suspended for 30 min at room temperature allowing the dye complex to bind to the GAG in solution. The GAG-dye complexes were precipitated by centrifugation at 10,000 ×g for 10 min. The supernatants were discarded carefully by inverting and drying the tube content. One ml of blyscan dissociation reagents was added to the tube, mixed by vortex and suspended for 15 min in room temperature releasing the bound dye for color analysis. Absorbance was read in a spectrophotometer at 656 nm, using water as blank. A linear standard curve was produced for the sulfated GAG standards. Total GAG of the samples was then determined and normalized to DNA content.

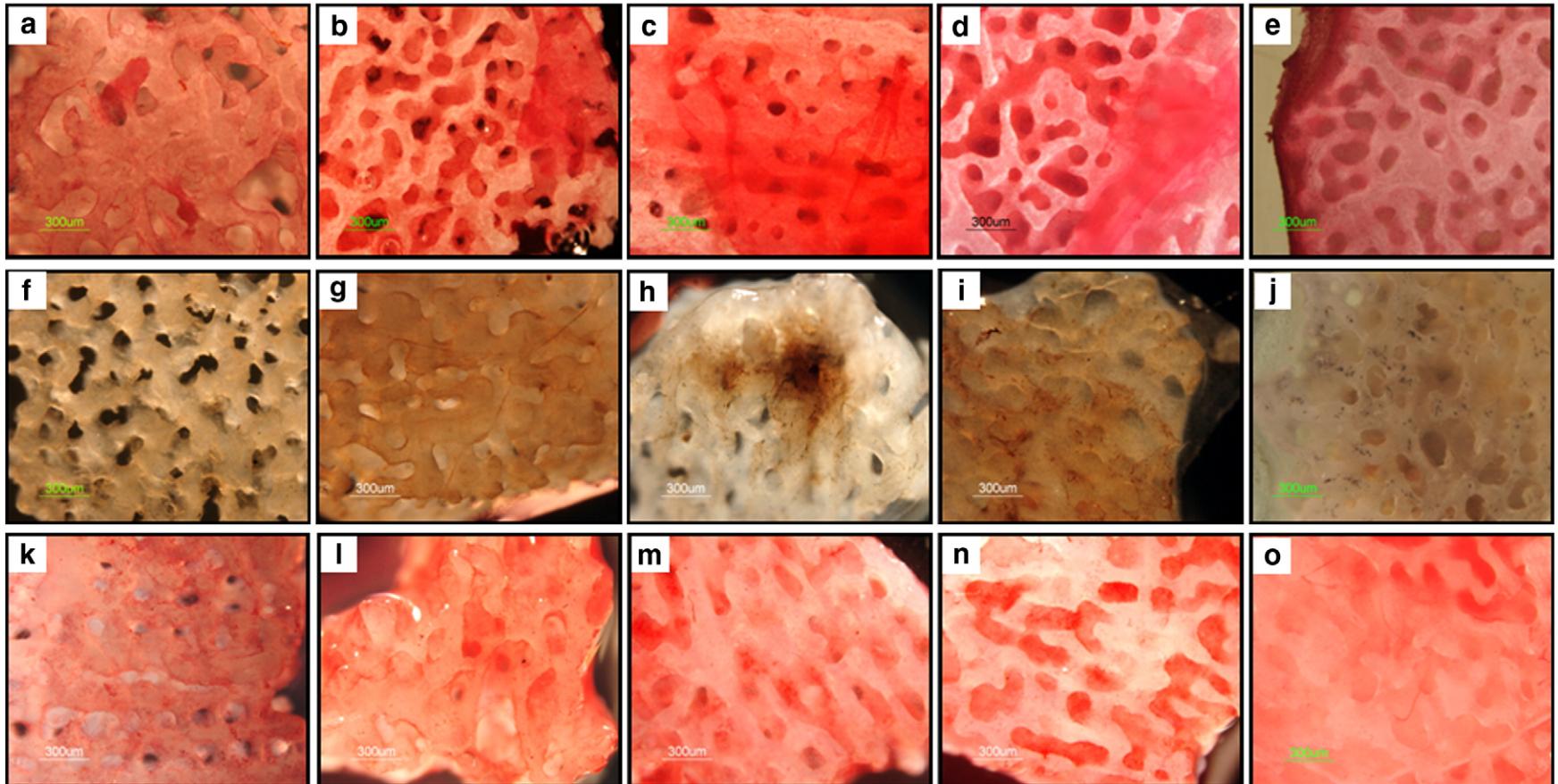
DNA concentration was determined using PicoGreen®dsDNA quantification kit (Molecular Probes Inc., USA). Fifty µl of papain digested sample was transferred to a 96 well fluorescence plate. One hundred µl of 1×TE solution (20 mM Tris-HCl, 20 mM EDTA, pH 7.5) was added to each well, followed by 150 µl of the Pico Green fluorescent double stranded DNA binding dye solution (diluted 1:200 with 1×TE solution) and incubated at room temperature, for 5 min in dark conditions. Excitation at 480 nm and emission at 520 nm was read using a fluorometer (Cary Eclipse Fluorescence spectrometer;



**Fig. 1.** SEM micrographs of chondrocytes cultured on MIL and POR scaffolds after 7 (a and d), 14 (b and e) and 21 (c and f) days post seeding, respectively. Rounded cells connected with fibers and embedded in the ECM shown in a and d (white arrows) and tissue covered the scaffolds with a smooth texture shown in b-f (white stars).



**Fig. 2.** Safranin O (a–e), von Kossa (f–j) and oil red O (k–o) staining of chondrocytes seeded on MIL scaffolds after 7 (a, f and k), 14 (b, g and l), 21 (c, h and m), 28 (d, i and n) and 42 (e, j and o) days post seeding.



**Fig. 3.** Safranin O (a–e), von Kossa (f–j) and oil red O (k–o) staining of chondrocytes seeded on POR scaffolds after 7 (a, f and k), 14 (b, g and l), 21 (c, h and m), 28 (d, i and n) and 42 (e, j and o) days post seeding.

VARIAN). Total DNA content was measured by plotting sample fluorescence values against a linear standard curve produced using dilutions of bacteriophage lambda DNA supplied with the kit.

### 2.7. Expression of genes encoding molecular markers by quantitative real time polymerase chain reaction (qPCR)

#### 2.7.1. RNA extraction

Total RNA of tissue culture on biomatrices was extracted using Tri-Reagent solution (MRC, USA), according to the single step acid-phenol guanidinium extraction method described by Chomczynski and Sacchi, 1987 [35]. Intact RNA integrity was tested on 1.7% agarose gel containing 0.25 µg/ml ethidium bromide. The concentration was quantified spectrometrically at 260 and 280 nm wavelengths (NanoDrop ND-1000, USA).

#### 2.7.2. Complementary DNA (cDNA)

2 µg of total RNA was converted into cDNA using EZ-First strand cDNA synthesis Kit (Biological Industries, Israel) according to the manufacturer protocol. At the end of the procedure the cDNA was diluted to a concentration of 20 ng/µl.

#### 2.7.3. qPCR

Gene specific primers were designed using the primer express™ software (V2.0, Applied Biosystems, USA), thus one of the paired primers was placed at the junction between two exons. The oligonucleotides were checked using PubMed Nucleotide BLAST and purchased from MWG (Germany). The following mouse-specific primer set were used (forward/reverse): SOX9, ACCAGTACCCG-CATCTGCAC/CTCGITCAGCAGCCTCCAG; GAPDH, CCTGGAGAACCTGC-CAAGTAT/GATGCCCTGCTTCACCACCTT.

Measuring of specific mRNA expression were done using SYBER green chemistry and monitored by an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). For each primer an optimal concentration was determined, which was sufficient for gene amplification without getting primer dimmers. The quantity of cDNA for each gene was determined according to standard curve at the accepted value after 25 cycles, which were required to reach threshold fluorescence signal level ( $C_t \approx 25$ ) and similar efficiency (appear as resembled slope in the standard curve). A 20 µl mixture of samples containing 10 µl platinum® Syber® green qPCR super mix-UDG with ROX (Invitrogen, USA), 5 µl of cDNA and complete amount of diethyl pyrocarbonate (DEPC) treated with double DW were prepared. For polymerase activation the samples were heated to 95 °C for 15 min. DNA was then amplified for 40 cycles of 95 °C for 15 min followed by 60 °C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as a reference gene. The expression levels were exhibited relative to the expression of the gene in the fourth day of the experiment. All analyses were preformed at least in duplicate and two independent experiments were performed.

### 2.8. Statistical analysis

GAG and DNA content from five different scaffolds were measured at each time point. Values of GAG content (normalized to DNA) are expressed as means ± standard deviation (SD) of the mean. Statistical analysis was performed by Students *t*-test.  $p < 0.05$  was considered significant.

## 3. Results

### 3.1. Chondrocyte culture

Chondrocytes were seeded as described above, on POR and MIL scaffolds and cultured for six weeks. The morphology of the tissue during the first three weeks of culture is shown in Fig. 1. No mor-

phological differences between the MIL and POR cultures were observed in this period. At the end of the first week of culture the cells were rounded and connected with fibers embedded in the ECM (white arrows). From the second week of culture onward tissue covered the scaffolds with a smooth texture (stars).

Figs. 2 and 3 show the histological analyses for chondrocytes seeded on MIL and POR, respectively. After the first week of culture, safranin O staining encircled the cells seeded on both biomatrices, while von Kossa and oil red showed no evidence of staining. This implies the presence of GAG molecules in the ECM of the tissue and no formation of bone nodules or fat aggregates. There was an extensive increase in the safranin O observation till the third week post seeding on both type of matrix, although it seems that the expression on POR was higher. After four and six weeks of culture the observed expression of safranin O decreased. At the third week of culture, von Kossa staining became evident, on MIL and POR. However, after four and six weeks post seeding, the appearance on POR was neglected while on MIL it was observed. This change in staining pattern suggests a reduction in GAG production and the beginning of a calcification process. The oil red O staining remained constant at low intensity of staining, throughout the six weeks of culture in either the seeded MIL or POR biomatrices.

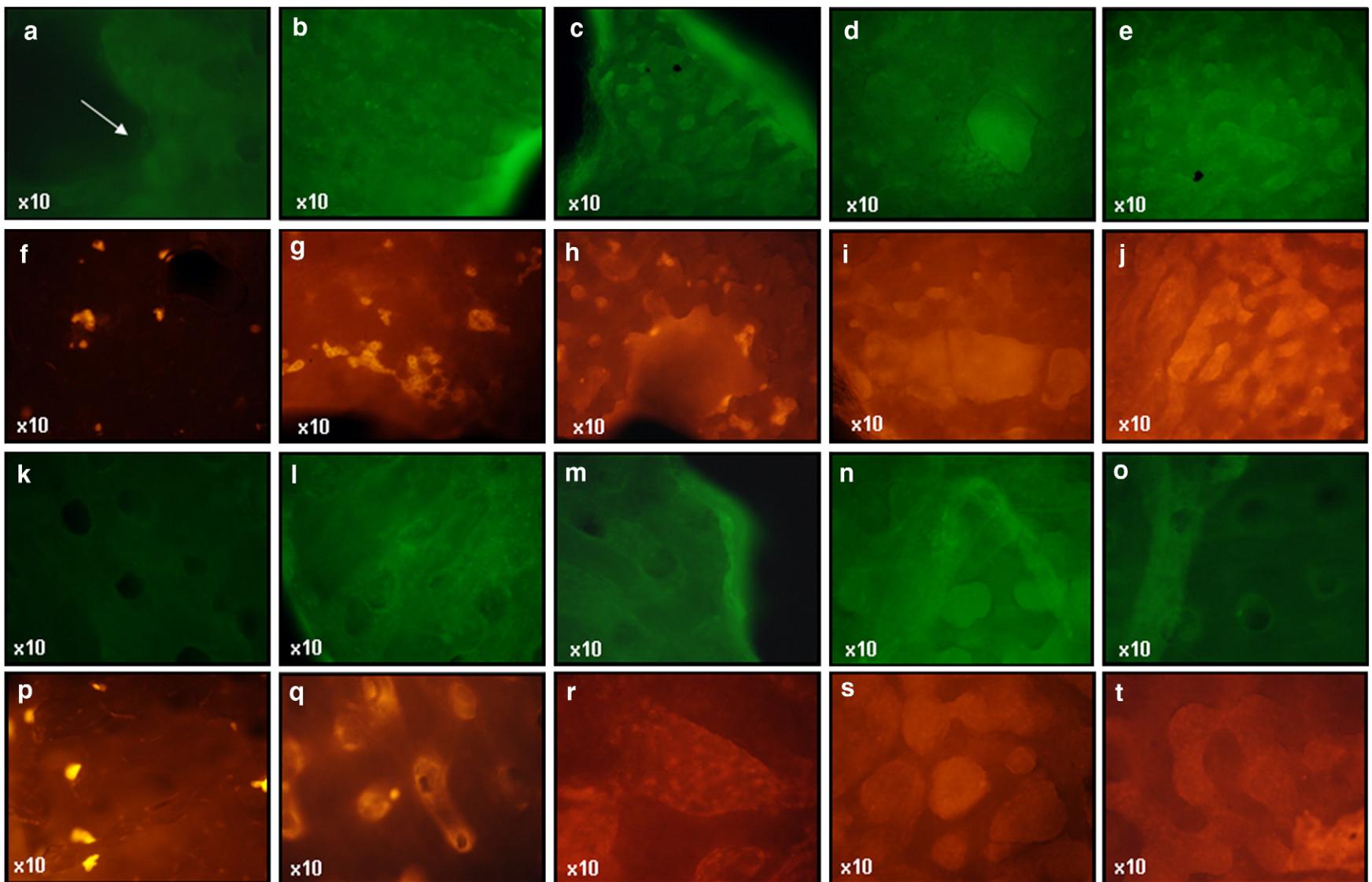
The expression of collagen types I and II was examined with immunofluorescence staining (Fig. 4). In the first week of culture, there was no detection of collagen type I on either matrix type (Fig. 4a and k). However, scattered areas of collagen type II were observed (Fig. 4f and p). During the second and the third weeks of culture, the areas of collagen type II staining expanded over the matrices (Fig. 4g, h, q and r) and, there are more expanded areas on POR than on MIL. At these time points the appearance of collagen type I was low on both matrices. Low levels of collagen type I with increased levels of collagen type II are characteristic of chondrocytes secreted ECM. During the last weeks of the study a trend toward decreasing observation of collagen type II (Fig. 4i, j, s and t) and increasing observation of collagen type I (Fig. 4d, e, n and o) were seen, implies on chondrocytes maturation.

The GAG content of chondrocytes seeded on MIL and POR scaffolds in the six weeks of culture is shown in Fig. 5. In both matrices a significant increase in GAG content was seen during the first two weeks of culture ( $p < 0.05$  for both matrices) followed by a decline during the final time points of the study. This trend correlated with the histological staining evidence which also demonstrated increased in GAG levels till the third week of culture followed by decreased over time. In comparison, the GAG content of the POR scaffolds was slightly higher than the MIL scaffolds during the first three weeks of the experiment; however, this difference was not found to be statistically significant ( $p > 0.05$ ). After the spike in GAG content seen at week two, the GAG content of both scaffold types remained constant and similar to each other in the remaining time points of the study.

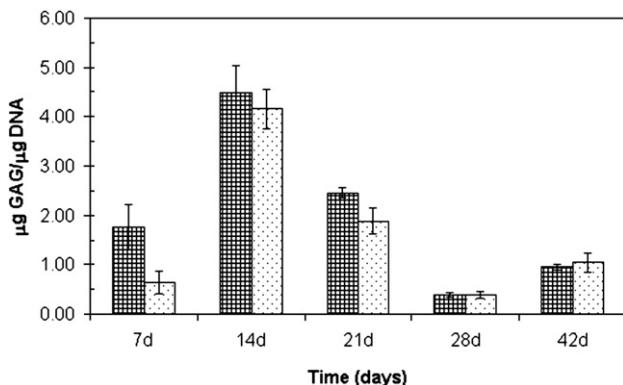
### 3.2. MSCs culture

As described in Methods, the culture medium for the MSCs was supplemented with TGF-β1 and IGF-I for the induction of chondrogenesis and maintenance of the chondrocytic phenotype during the culture period. The additive or synergistic affects of these supplements were examined and it was found that the combination of TGF-β1 and IGF-I is better than either alone for the chondrogenesis of MSCs seeded on POR (data not shown).

SEM micrographs indicating the morphology of MSCs seeded on MIL and POR, during the first three weeks of culture are shown in Fig. 6. The tissue on MIL covered the matrix with a smooth, uniform texture with periodic rounded cells embedded in the tissue (Fig. 6a, b and c). The tissue covering the POR matrix after two weeks of culture was rough in appearance with cells more spherical in shape connected by fibers (Fig. 6d and e). After three weeks of culture, the tissue on the



**Fig. 4.** Collagen type I (a–e and k–o) and collagen type II (f–j and p–t) immunofluorescence micrographs of chondrocytes seeded on MIL (a–j) and POR (k–t) scaffolds after 7 (a, f, k and t), 14 (b, g, l and q), 21 (c, h, m and r), 28 (d, i, n and s) and 42 (e, j, o and t) days post seeding.



**Fig. 5.** GAG content (normalized to DNA content) of chondrocytes seeded on POR (squares) and MIL (dots). Columns represent mean and error bars represent SD.

POR matrix looked much smoother (Fig. 6f), resembling the tissue on the MIL scaffold at the same time point.

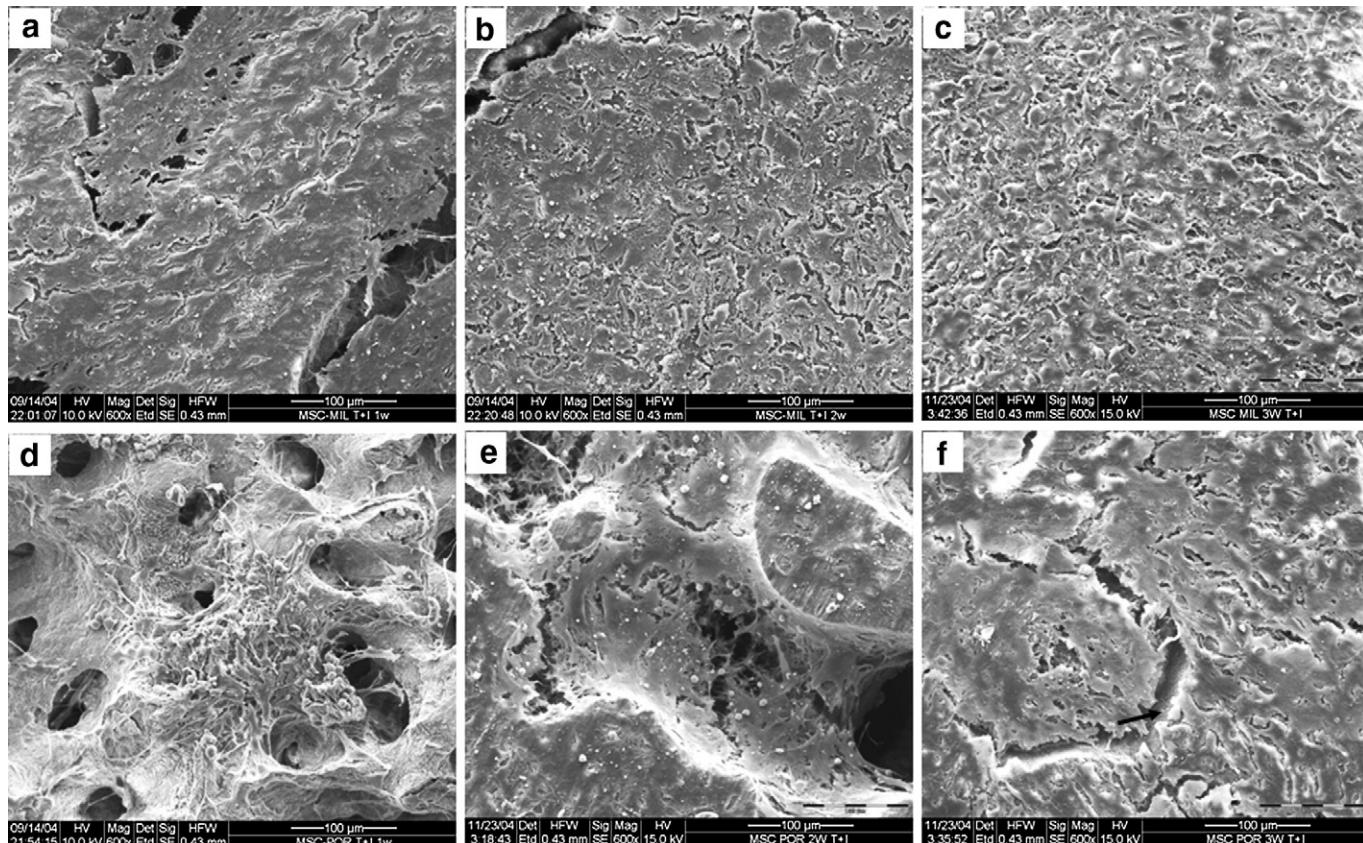
The differentiation of the MSCs seeded on MIL and POR was examined (Figs. 7 and 8, respectively) using safranin O, von Kossa and oil red O histochemical staining. Safranin O staining was detected one week post seeding on the MIL and POR matrices. Neither scaffold exhibited von Kossa or oil red staining at this time point. The lack of von Kossa staining along with the positive staining for GAG implies a shift toward chondrogenesis of the MSCs. After the second and the third weeks of culture, a strong GAG staining by safranin O was observed for both cultures. Slight indications of von Kossa staining appeared by the second week of culture for the culture on MIL and POR scaffolds. The appearance of von Kossa staining indicates of further differentiation of cells and the beginning of an ossification process. After the fourth and sixth weeks of culture safranin O staining began to fade while von Kossa staining increased. These results are

similar to the behavior of chondrocytes after the third week of culture, where the loss of the chondrocytic phenotype and further differentiation was observed along with the appearance of bone nodules in the tissue. Low expression of Oil red O staining for adipose tissue observed from the culture on both marine origin scaffolds, throughout the study.

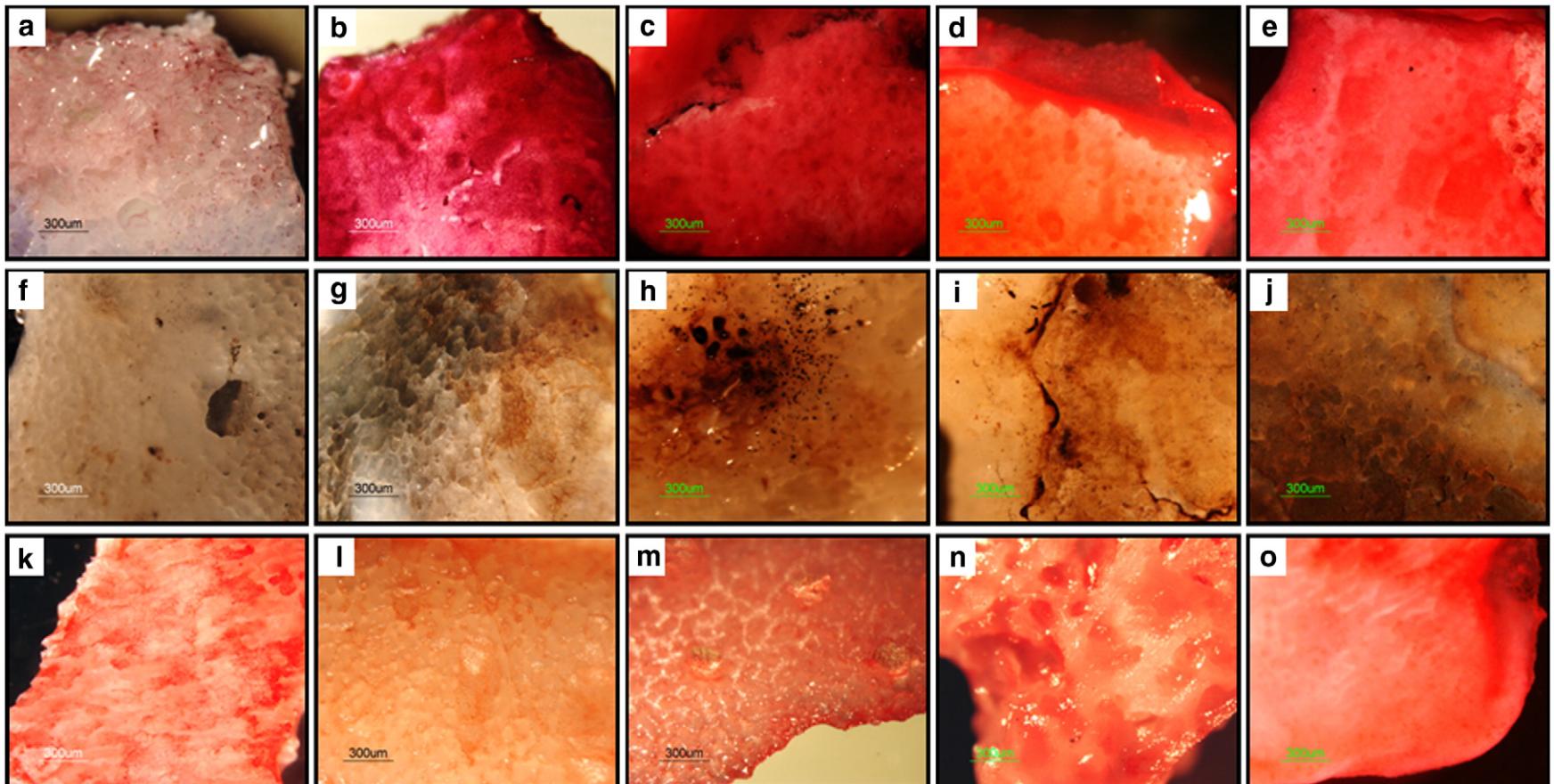
Immunofluorescence analyses revealed low accumulation of collagen type II (Fig. 9f and p) on MIL and POR after one week of culture. No collagen type I expression was seen at this time point (Fig. 9a and k). After two weeks of culture, collagen type I remained low with extensive expression of collagen type II (Fig. 9q) for cells cultured on POR. On MIL collagen type I expression was observed together with collagen type II (Fig. 9b and g) at this time point. At each of the remaining time points expression of both types of collagen was detected in cultures of each scaffold type. These results are in agreement with the histochemical analyses described above: initial chondrogenesis of MSCs at the beginning of the culture period followed by further differentiation after two weeks of culture.

A quantitative measure of GAG content in tissues cultured on MIL and POR is shown in Fig. 10. There were no statistically significant ( $p>0.05$ ) differences in GAG content on both matrices during the study period. GAG content was maximal for both scaffold types after the first week of culture and diminished at the second week of culture. After three weeks of culture, there was a slight increase in GAG content that remained constant at the later two time points.

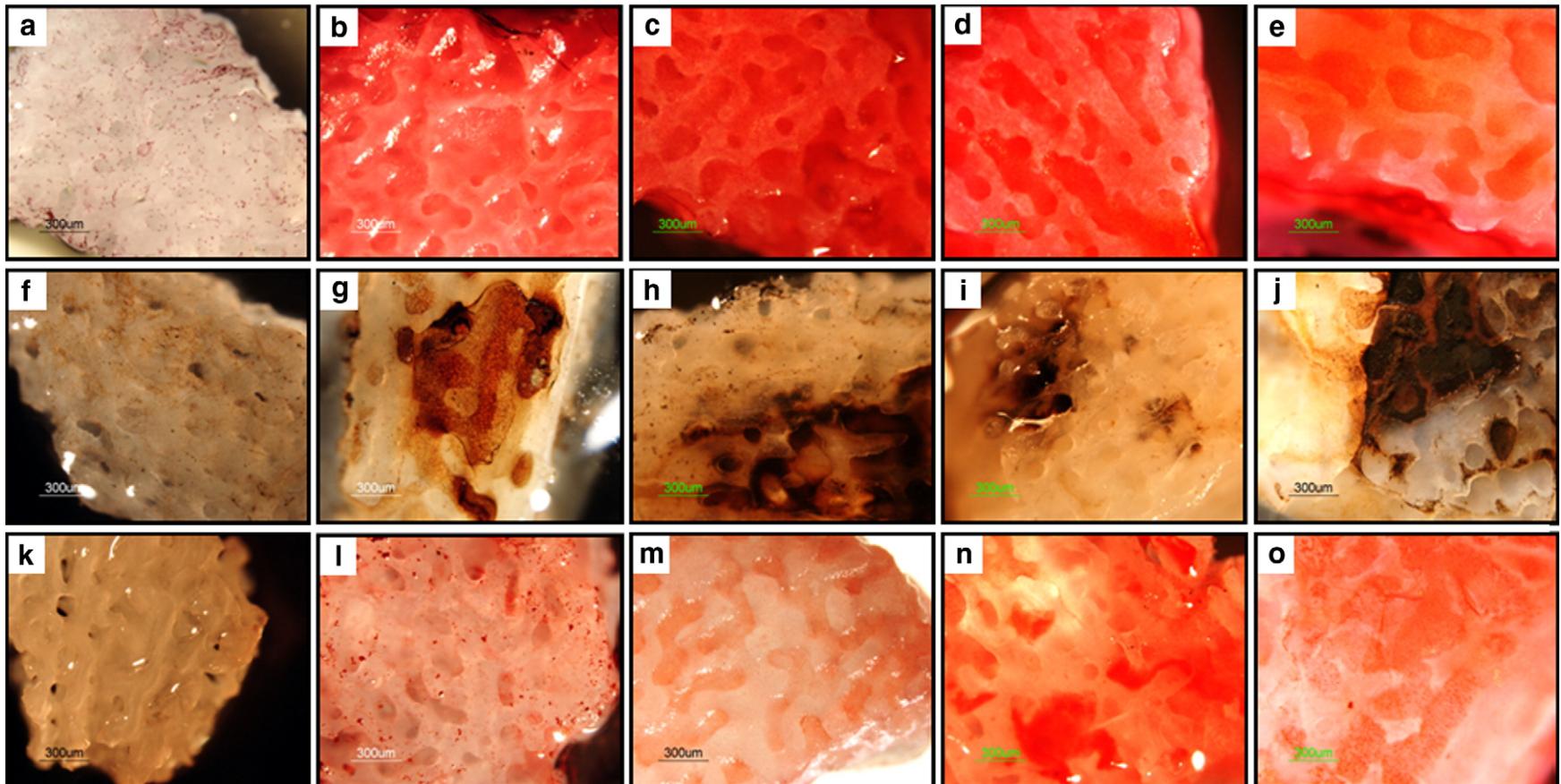
A quantitative-PCR used for detection of SOX9. Fig. 11 exhibited the changing in the expression of it during six weeks of MSCs culture on MIL and POR. A comparison between the first and the second week of MSCs cultured on MIL revealed to significant increase ( $p<0.01$ ) in the expression of SOX9. However, after three weeks of culture onward SOX9 was not detected at this culture. In contrast, SOX9 expressed during the whole six weeks of culture on POR. At the first two weeks of culture its level was increased (not significantly) followed by non



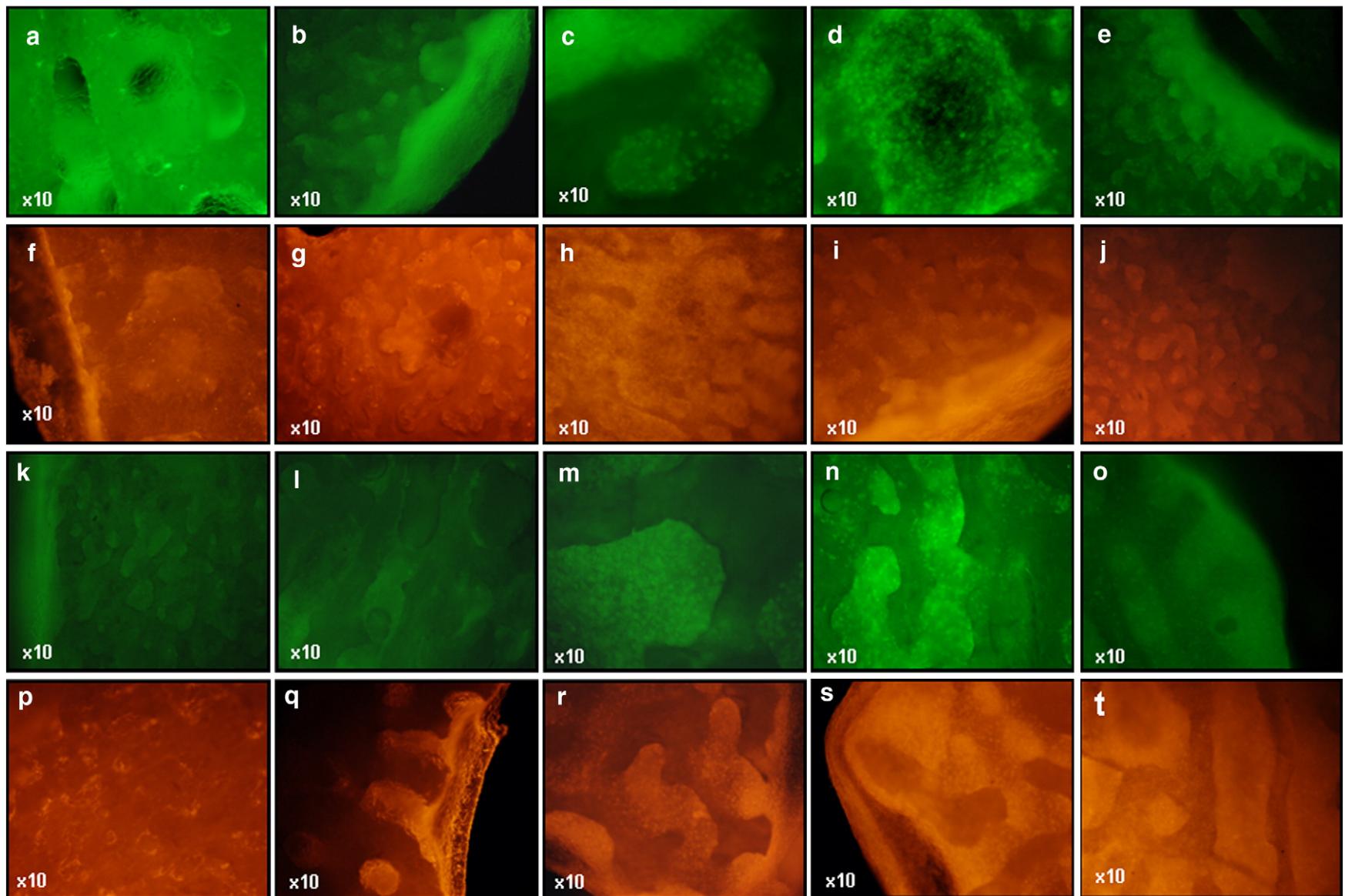
**Fig. 6.** SEM micrographs of MSCs cultured on MIL and POR scaffolds after 7 (a and d), 14 (b and e) and 21 (c and f) days post seeding, respectively.



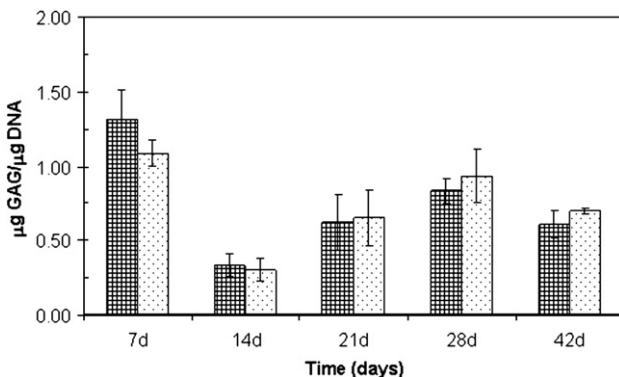
**Fig. 7.** Safranin O (a–e), von Kossa (f–j) and oil red O (k–o) staining of MSCs seeded on MIL scaffolds after 7 (a, f and k), 14 (b, g and l), 21 (c, h and m), 28 (d, i and n) and 42 (e, j and o) days post seeding.



**Fig. 8.** Safranin O (a–e), von Kossa (f–j) and oil red O (k–o) staining of MSCs seeded on POR scaffolds after 7 (a, f and k), 14 (b, g and l), 21 (c, h and m), 28 (d, i and n) and 42 (e, j and o) days post seeding.



**Fig. 9.** Collagen type I (a–e and k–o) and collagen type II (f–j and p–t) immunofluorescence micrographs of MSCs seeded on MIL (a–j) and POR (k–t) scaffolds after 7 (a, f, k and t), 14 (b, g, l and q), 21 (c, h, m and r), 28 (d, i, n and s) and 42 (e, j, o and t) days post seeding.



**Fig. 10.** GAG content (normalized to DNA content) of MSCs seeded on POR (squares) and MIL (dots). Columns represent mean and error bars represent SD.

significant decreased ( $p>0.05$ ). The increased level of SOX9 on MIL was higher than the increased level on POR after two weeks of culture but the difference between the increased values was not significant. The appearance of SOX9 matched the previous results indicated of MSCs chondrogenesis followed by further differentiation and loose of chondrocytes phenotype after two weeks of culture.

#### 4. Discussion

A promising direction toward tissue engineering of articular cartilage is based on the *ex-vivo* formation of tissue for eventual use in clinical repair of cartilage defects [36]. In this process, it is important to optimize conditions related to cell types, scaffolds and environmental cues to create a suitable tissue and preserve its articular cartilage phenotype. In this study we examined crystalline matrices of marine origin as candidates for scaffolds in cartilage tissue engineering.

Cell adhesion, proliferation and complete tissue coverage of scaffold matrix are key components in engineering any tissue. Therefore, many studies have been carried out in order to tackle the problem of cell adhesion to scaffolds and the expansions of tissue around the matrices after seeding [1,37]. Some cases demand further steps or actions in order to seed cells on the scaffolds for example: seeding procedure on collagen matrices and PGA requires a dynamic seeding created by stirring the media in flasks [38,39]. This complicated procedure increases the culture time. Moreover, these techniques, which do not yield complete attachment of cells to the material, require a high density of cells. The need for a high density of chondrocytes is a disadvantage when the source of the cells is autologous or allogeneic and there is a need to sacrifice healthy tissue to repair the injury. Our study shows that both chondrocytes and MSCs adhere rapidly to POR and MIL and the seeding procedure does not require high cellular densities for fast development of tissue. These results could be advantageous when the need of creation engineered implant is urgent.

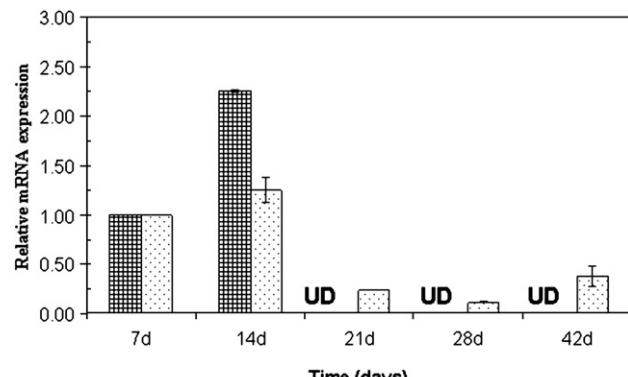
Chondrocytes seeded on POR and MIL presented a typical spherical morphology on both matrices as commonly reported [6]. Moreover, POR and MIL scaffolds provided a favorable environment for chondrocyte development and allowed direct cell-biomaterial and cell-cell interactions, expressed by high capacity of adhesion and proliferation, from the first week of culture and thus support seeding of extremely low cell densities. It should be noted that in both cases adhesion to the biomaterials of chondrocytes and MSCs seeded on the POR and MIL took place spontaneously with no additional physical means or biochemical supplements. We assume that the crystalline structure of aragonite along with the architectural configuration and surface parameters of this composite biomaterial allow these interactions via direct membrane receptors and provide appropriate environment for proliferation and development of the tissue on the

scaffolds. These findings are supported by previous study demonstrated that the amount of human MSCs incorporated onto coral is higher than the amount of those incorporated onto a smooth surface calcite form [31].

In the case of MSCs, there is evidence for chondrocyte-like cells with spherical morphology at the beginning of culture, especially on POR during the first week. However, most of the cells on MIL lost the chondrocytic morphology after the first week of culture and tended toward fibrochondrocyte morphology. After three weeks of culture, similar changes in the tissue morphology were observed on POR. Fibrocartilage is composed primarily of collagen type I and proteoglycans such as versican, which are characteristic of mesenchymal dermis. The ECM of fibrocartilage provides a significantly lower biomechanical compressive strength in comparison with hyaline cartilage. Lower biomechanical strength creates an undesirable situation that could lead to further cartilage injury instead of healing [36].

GAG molecules connected to a hyaluronan core in the ECM are a characteristic hallmark of chondrocytes [6]. Therefore, we suggest that the appearance of GAG molecules implies the presence of hyaluronan as well. GAG and hyaluronan are involved in many cellular processes such as cell adhesion, proliferation, signaling, motility [40,41] and support tissue's biomechanical properties [42]. The evidence of these molecules at the beginning of MSCs culture indicates that the POR and MIL scaffolds initially provide a good environment for chondrogenesis. However, long term observations indicated on further MSCs differentiation towards hypertrophic chondrocytes. We assume that the signals transmitted from the natural marine origin biomatrices are very strong, therefore the influence of the growth factors diminished.

Tissue development of cartilage begins with condensation of MSCs followed by differentiation into chondrocytes. This process could continue with further differentiation to hypertrophic chondrocytes and the beginning of the mineralization process followed by development of osteoblasts and bone formation. The whole process is called endochondral ossification [8,43,44]. Reiter et al., presented a model of primary monolayer chondrocyte culture that undergoes spontaneous chondrogenesis, resembling the endochondral ossification process [45]. We suggest that the development of cartilage tissue from chondrocytes on POR or MIL more closely resembles the native endochondral ossification process. During the first three weeks of culture the expression of GAG and collagen type II were high, as it should be in chondrocytes. After the first period of culture, the expression of collagen type II decreased and the expression of collagen type I increased. Further culture led to appearance of phosphorous in the tissue, shown by expression of von Kossa staining, which implies bone mineralization. It is interesting to note that this process was accelerated when chondrocytes were seeded on MIL with a time shift of about one week between the two cultures. The endochondral



**Fig. 11.** mRNA expression of SOX9 in MSCs seeded on POR (squares) and MIL (dots) fold changes  $\pm$  SD, relative to the expression at the seventh day of culture. UD represent undetected results.

ossification process on POR and MIL was better demonstrated with MSCs. This process began with differentiation of MSCs into chondrocytes and continued with further differentiation to osteoblasts. The mineralization process of MSCs appeared on MIL before it appeared on POR, and it was similar to the behavior of chondrocytes. Furthermore, the transcription factor SOX9 is essential for converting MSCs into chondrocytes [43]. The behaviour of SOX9 on MIL and POR emphasizes the appearance of chondrocytes phenotype at the first two weeks of culture, on both matrices, and loose of chondrocytes characteristics after three weeks of culture. Again, a comparison between the matrices exhibited an acceleration of chondrocytes phenotype loose on MSCs seeded on MIL. The reason for the acceleration in the mineralization process on MIL could be due to different densities between POR and MIL, or could be due to different topology and the organic components found in the exoskeleton of these species [33]. Although the crystalline microstructure of MIL (hydrozoa) is similar to those of the scleractinian corals (Anthozoa), the architectural and/or biochemical character of its organic components may be somewhat different [46]. The mineralization of the cartilage tissue is not a desirable process especially when it appears after two or three weeks of culture.

These coralline materials exhibit some desirable characteristics such as mechanical integrity for culture manipulation, prompt and good cell adhesion, suitable conditions appropriate for cell proliferation and early cartilage development. We suggest that cells in contact with these 3D biomatrices having an aragonite crystalline structure, exploit calcium ions from the biomaterial leading to the prompt adhesion while affecting proliferation and differentiation. This is in agreement with recent studies dealing with adhesion and behavior of different type of cells seeded on coralline biomaterials [46–48]. However, this study's results suggest that MIL has a stronger osteogenic capacity than POR as it enhances *ex-vivo* ossification of MSCs in a faster manner than POR. These results are further supported by other observations in our laboratory [46]. The osteoinductivity of POR and in particular of MIL could be an obstacle for using these materials in the repair of articular cartilage. However, these may be overcome by optimization of the correct growth factors (time dependant, ratios and dose) and further induction factors, considered to be causative toward the chondrogenic phenotype, such as direct compression or hydrostatic pressure.

## Acknowledgments

The authors wish to thank Aviva Kiryati for the expert assistance with SEM analysis. This work was partially supported by the Binational Science Foundation (BSF) # 2001045.

## References

- [1] V.R. Makarand, M. Sittinger, Trends Biotechnol. 20 (2002) 351.
- [2] L.J. Sandell, T. Aigner, Arthritis Res. 3 (2001) 107.
- [3] S.R. Goldring, M.B. Goldring, in: M.J. Seibel, S.P. Robins, J.P. Bilezikian (Eds.), *Dynamics of Bone and Cartilage Metabolism*, Academic press, San Diego, USA, 1999, p. 623.
- [4] R.G. LeBaron, K.A. Athanasiou, Biomaterials 21 (2000) 2575.
- [5] E. Darling, K.A. Athanasiou, J. Orthop. Res. 23 (2) (2005) 425.
- [6] J.C.Y. Hu, K.A. Athanasiou, in: Y.H. An, K.L. Martin (Eds.), *Handbook of Histology Methods for Bone and Cartilage*, Humana press Inc., NJ, USA, 2003, p. 73.
- [7] B. Johnstons, T.M. Hering, A.I. Caplan, V.M. Goldberg, J.U. Yoo, Exp. Cell Res. 238 (1998) 265.
- [8] J.E. Dennis, A.I. Caplan, in: S. Sell (Ed.), *Stem Cells Handbook*, Humana Press Inc., New Jersey, USA, 2004, p. 107.
- [9] A.I. Caplan, S. Bruder, Trends Mol. Med. 7 (2001) 259.
- [10] J.J. Minguell, A. Erices, P. Conget, Exp. Biol. Med. 226 (6) (2001) 507.
- [11] G.T. Sukhikh, V.V. Malaitsev, I.M. Bogdanova, I.V. Dubrovina, Bull. Exp. Biol. Med. 133 (2001) 103.
- [12] T. Fukumoto, J.W. Sperling, A. Sanyal, J.S. Fitzsimmons, G.G. Reinholz, C.A. Conover, S.W. O'Driscoll, Osteoarthr. Cartil. 11 (2003) 55.
- [13] G. Schmidmaier, B. Wildemann, T. Gabelin, J. Heeger, F. Kandziora, N.P. Haas, M. Raschke, Acta Orthop. Scand. 74 (5) (2003) 604.
- [14] A. Vats, N.S. Tolley, J.M. Polak, J.E. Gough, Clin. Otolaryngol. 28 (2003) 165.
- [15] B. Kinner, M. Spector, in: A. Atala, R.P. Lanza (Eds.), *Methods of Tissue Engineering*, Academic Press, California, USA, 2002, p. 317.
- [16] J.C. Hu, K.A. Athanasiou, Biomaterials 26 (2005) 2001.
- [17] L.E. Freed, G. Vunjak-Novakovic, R.J. Biron, D.B. Eagles, D.C. Lesnoy, S.K. Barlow, R. Langer, Biotechnology 12 (1994) 689.
- [18] A. Mikos, A. Thorsen, L. Czerwonka, Y. Boa, D. Winslow, J. Vacanti, R. Langer, Polymer 35 (1994) 1068.
- [19] J.S. Temenoff, A.G. Mikos, Biomaterials 21 (2000) 431.
- [20] A.M. Freyria, D. Cortial, M.C. Ronziere, S. Guerret, D. Herbage, Biomaterials 25 (2004) 687.
- [21] J.S. Pieper, P.M. van der Kraan, T. Hafmans, J. Kamp, P. Buma, J.L.C. van Susante, W.B. van den Berg, J.H. Veerkamp, T.H. van Kuppevelt, Biomaterials 23 (2002) 3183.
- [22] M.M. Stevens, H.D. Qanadilo, R. Langer, V. Prasad Shastri, Biomaterials 25 (2004) 887.
- [23] H.L. Ma, S.C. Hung, S.Y. Lin, Y.L. Chen, W.H. Lo, J. Biomed. Mater. Res. 64A (2003) 273.
- [24] J.K.F. Suh, H.W.T. Matthew, Biomaterials 21 (2000) 2589.
- [25] D.A. Grande, C. Halberstadt, G. Naughton, R. Schwartz, R. Manji, J. Biomed. Mater. Res. 34 (1997) 211.
- [26] R. Vago, D. Plotquin, A. Bunin, I. Sinelnikov, D. Atar, D. Itzhak, J. Biochem. Biophys. Methods 50 (2002) 253.
- [27] J. Hu, R. Fraser, J.J. Russell, B. Ben-Nissan, R. Vago, J. Mater. Sci. Technol. 16 (2000) 591.
- [28] H. Ohgushi, A.I. Caplan, J. Biomed. Mater. Res. 48 (1999) 913.
- [29] C. Demers, C.R. Hamdy, K. Corsi, F. Chellat, M. Tabarizian, L. Yahia, Bio-med. Mater. Eng. 12 (2002) 15.
- [30] D. Green, D. Walsh, S. Mann, R.O.C. Oreffo, Bone 30 (6) (2002) 810.
- [31] J.C. Fricain, R. Bareille, F. Ulysse, B. Dupuy, J. Amedee, J. Biomed. Mater. Res. 42 (1998) 96.
- [32] J. Vuola, T. Bohling, J. Kinnunen, E. Hirvensalo, S. Asko-Seljavaara, J. Biomed. Mater. Res. 51 (2000) 117–122 2000.
- [33] D. Dahan, Microstructure and skeletal architecture of aragonite based biomaterials of marine origin. [M.Sc. thesis], Department of material engineering, Ben-Gurion University of the Negev, Beer-Sheva, Israel, 2003.
- [34] L. Abramovitch-Gottlib, D. Katoshevski, R. Vago, J. Biochem. Biophys. Methods 50 (2002) 245.
- [35] P. Chomczynski, N. Sacchi, Anal. Biochem. 162 (1) (1987) 156.
- [36] J.M. McPherson, R. Tubo, in: R.P. Lanza, R. Langer, J. Vacanti (Eds.), *Principles of Tissue Engineering*, Academic press, California, USA, 2000, p. 697.
- [37] W.M. Saltzman, in: R.P. Lanza, R. Langer, J. Vacanti (Eds.), *Principles of Tissue Engineering*, Academic press, California, USA, 2000, p. 221.
- [38] L.E. Freed, G. Vunjak-Novakovic, in: R.P. Lanza, R. Langer, J. Vacanti (Eds.), *Principles of Tissue Engineering*, Academic press, California, USA, 2000, p. 143.
- [39] K.J. Gooch, T. Blunk, D.L. Courter, A.L. Sieminski, G. Vunjak-Novakovic, L.E. Freed, Tissue Eng. 8 (2002) 591.
- [40] B.P. Toole, J. Intern. Med. 242 (1997) 35.
- [41] M. Cohen, E. Klein, B. Geiger, L. Addadi, Biophys. J. 85 (2003) 1996.
- [42] T. Hardingham, in: M.J. Seibel, S.P. Robins, J.P. Bilezikian (Eds.), *Dynamics of Bone and Cartilage Metabolism*, Academic press, California, USA, 1999, p. 71.
- [43] H.M. Kronenberg, Nature 423 (2003) 332.
- [44] A.M. DeLise, L. Fischer, R.S. Tuan, Osteoarthr. Cartil. 8 (2000) 309.
- [45] I. Reiter, M. Tzukerman, G. Maor, Bone 31 (2002) 333.
- [46] L. Abramovitch-Gottlib, S. Geresh, R. Vago, Tissue Eng. 12 (2006) 729.
- [47] Z.R. Birk, L. Abramovitch-Gottlib, I. Margalit, M. Aviv, E. Forti, S. Geresh, R. Vago, Tissue Eng. 12 (2006) 21.
- [48] B. Shany, R. Vago, D. Baranes, Tissue Eng. 11 (2005) 585.