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## Comparative Chondrogenesis of Human Cell Sources in 3D Scaffolds

R. Seda Tıǧlı<sup>1,2</sup>, Sourabh Ghosh<sup>1</sup>, Michael M. Laha<sup>1</sup>, Nirupama K. Shevde<sup>3</sup>, Laurence Daheron<sup>4</sup>, Jeffrey Gimble<sup>5</sup>, Menemşe Gümüşdereliolu<sup>2</sup>, and David L. Kaplan<sup>1,\*</sup>

<sup>1</sup> Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA

<sup>2</sup> Hacettepe University, Chemical Engineering Department, 06800, Beytepe-Ankara, TURKEY

<sup>3</sup> WiCell Research Institute, Madison, WI 53707-7365, USA

<sup>4</sup> Massachusetts General Hospital, Center for Regenerative Medicine and Technology, 185 Cambridge Street, Boston, MA 02114, USA

<sup>5</sup> Pennington Biomedical Research Center, 6400 Perkins Rd, Baton Rouge, LA 70808, USA

### Abstract

Cartilage tissue can be engineered by starting from a diversity of cell sources, including stem-cell based and primary cell-based platforms. Selecting an appropriate cell source for the process of cartilage tissue engineering or repair is critical and challenging due to the variety of cell options available. In this study, cellular responses of isolated human chondrocytes, human embryonic stem cells and mesenchymal stem cells (MSCs) derived from three sources, human embryonic stem cells, bone marrow and adipose tissue, were assessed for chondrogenic potential in 3D culture. All cell sources were characterized by FACS analysis to compare expression of some surface markers. The cells were differentiated in two different biomaterial matrices, silk and chitosan scaffolds, in the presence and absence of bone morphogenetic protein 6 (BMP-6) along with the standard chondrogenic differentiating factors. Embryonic stem cells derived MSCs showed unique characteristics with preserved chondrogenic phenotype in both scaffolds with regard to chondrogenesis, as determined by real time RT-PCR, histological and microscopic analyses. After 4 weeks of cultivation, embryonic stem cells derived MSCs were promising for chondrogenesis, particularly in the silk scaffolds with BMP-6. The results suggest that cell source differences are important to consider with regard to chondrogenic outcomes and with the variables addressed here, the human embryonic stem cells derived MSCs were the preferred cell source.

### Keywords

chondrocytes; stem cells; silk; chitosan; chondrogenesis; scaffold

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\*Address for correspondence: Dr. David L. Kaplan, David.Kaplan@tufts.edu Tel: +1617 627 3251 Fax: +1617 627 3231.  
R. Seda Tıǧlı: stigli@hacettepe.edu.tr, Tel: +1617 627 2580, Fax: +1 617 627 3231  
Sourabh Ghosh: ghoshsourabh@yahoo.com, Tel: +1617 627 2580, Fax: +1 617 627 3231  
Michael M. Laha: Michael.Laha@tufts.edu, Tel: +1617 627 2580, Fax: +1 617 627 3231  
Nirupama K. Shevde: RShevde@wicell.org, Tel: +1608 262 8231, Fax: +1608 263 7609  
Laurence Daheron: ldaheron@partners.org, Tel: +1617 724 0237, Fax: +1617 724 2662  
Jeffrey Gimble: Jeffrey.Gimble@pbrc.edu, Tel: +1225 763 3171, Fax: +1225 763 0273  
Menemşe Gümüşdereliolu: menemse@hacettepe.edu.tr, Tel: +90312 297 7447, Fax: +903122992124

## 1. Introduction

Severe damage to cartilage tissue caused by developmental abnormalities, trauma, or aging-related degeneration such as osteoarthritis result in disability and extensive pain. Adult cartilage tissue has limited self-repair capacity due to the sparse distribution of highly differentiated, non-dividing chondrocytes, slow matrix turnover, low supply of progenitor cells and lack of vascular supply (Wang *et al.* 2005). The inability of cartilage for self-repair and the lack of protocols that can reproducibly regenerate durable articular surfaces provide the rationale for the development of new treatment options based on tissue-engineered cartilage approaches, since current treatment methods for cartilage damage are often not sufficient to restore normal physiological function (Lohmander 2003; Tuan *et al.* 2003). Successful cartilage tissue engineering requires cells capable of undergoing chondrogenic differentiation upon treatment with appropriate biochemical and physical regulatory factors and biomaterial scaffolds capable of providing a favorable environment for chondrogenic cell growth and new cartilage-specific ECM formation (Langer and Vacanti 1993).

Chondrocytes are a major cell source used to generate engineered cartilage tissue and are commonly isolated from articular cartilage tissues. These cells have been used clinically to treat full-thickness cartilage defects (Brittberg *et al.* 1994). Developmentally, chondrocytes are derived from a common cell source, mesenchymal stem cells (MSCs) (Caplan 1991). In contrast to adult chondrocytes, MSCs are easier to obtain and can be manipulated for multiple passages before losing differentiation potential. MSCs have previously been derived from bone (Sottile *et al.* 2002), bone marrow (Pittenger *et al.* 1999), muscle (Mastrogiacomo *et al.* 2005), and fat (Zuk *et al.* 2001), and are capable of multi-lineage differentiation. Previous reports have shown that MSCs derived from bone marrow and adipose stromal cells (ASCs) derived from adipose tissue, provide attractive cell sources for cartilage tissue engineering in vitro and in vivo (Wakitani *et al.* 1994; Im 2005; Wang *et al.* 2005; Jin *et al.* 2007; Kisiday *et al.* 2008) but their use in clinical trials for applications in humans remains in early stages (Wakitani *et al.* 2002). The *ex vivo* expansion of chondrocytes results in a loss of their phenotype (Homicz *et al.* 2002) and the self-renewal and proliferative capacity of MSCs decreases with the number of passages in culture and the age of the donor (Fehrer and Lepperdinger 2005). Embryonic stem cells (ESCs), pluripotent precursor cells with indefinite self-renewing ability (Wobus *et al.* 1984; Thomson 1998) are a potential source for cartilage tissue engineering because they provide an unlimited supply of cells that can be differentiated into chondrocytes. Previous reports have shown successful cartilage tissue formation by ESCs, although it is stated that differentiation protocol requires further improvements to achieve homogeneous differentiation and abolish teratoma formation in vivo (Koay *et al.* 2007; Jukes and Moroni L 2008). Another potential source of MSCs is human embryonic stem cell-derived multipotent cells, devoid of the above limitations of both ESCs and MSCs in tissue engineering applications, as well as having negligible risk of teratoma formation (Sze *et al.* 2007). However, until now only a limited number of studies have been performed with human embryonic stem cell-derived MSCs for tissue engineering (Hwang *et al.* 2006; Karp *et al.* 2006; Wu *et al.* 2006; Sze *et al.* 2007). Several studies were reported where different cell sources were screened for comparative outcomes in terms of chondrogenesis (Winter *et al.* 2003; Im *et al.* 2005; Park *et al.* 2006; Afizah *et al.* 2007; Yoshimura *et al.* 2007; Kisiday *et al.* 2008). Throughout these studies, mainly the chondrogenic potential of adipose tissue derived MSCs and bone marrow derived MSCs were compared in both pellet culture and 3D culture systems, and the results showed an inferior potential for the adipose tissue derived MSCs over bone marrow derived MSCs in terms of chondrogenesis (Winter *et al.* 2003; Im *et al.* 2005; Afizah *et al.* 2007; Kisiday *et al.* 2008). Rat MSCs derived from bone marrow, synovium, periosteum, adipose tissue and muscle were compared in vitro in pellet culture systems and the synovium-derived cells had the greatest potential for chondrogenesis (Yoshimura *et al.* 2007). In an in vivo study for the

comparison of MSCs derived from bone marrow, perichondrium/periosteum and fat of adult rats, the superiority of perichondrium/periosteum-derived cells and BMSC to cells isolated from fat with respect to forming hyaline cartilaginous tissue was reported (Park *et al.* 2006). However, it is evident that an extended comparison of cell sources in 3D culture systems is required for needs in the field of cartilage tissue engineering and cartilage repair.

There are a variety of biopolymers that can be used as scaffolds for cartilage tissue engineering, including poly(L-lactide)/poly( $\epsilon$ -caprolactone) (Zhao *et al.* 2004), polyglycolide (Freed *et al.* 1993) and poly(lactide-co-glycolide) (Sittinger *et al.* 1996). However, these materials are problematic because they can induce inflammation due to elevated acidity during polymer hydrolysis, local tissue degeneration, processing difficulties may lead to inconsistent biodegradation rates and tissue response profiles or degradation profiles do not match the rate of tissue regeneration (Athanasίου *et al.* 1996; Suh 1998). Naturally occurring polymers as scaffolds offer options for cartilage tissue engineering due to biocompatibility, biodegradability, low toxicity of degradation byproducts, and plasticity in processing into a variety of material formats (Velema and Kaplan 2006). Chitosan is a partially deacetylated derivative of chitin and to some extent mimics the chemistry of articular cartilage components like glucosaminoglycans (GAGs) and hyaluronic acid (Sechriest *et al.* 2000). Thus, chitosan has been used as a scaffold for cartilage repair (Lahiji *et al.* 2000; Sechriest *et al.* 2000; Suh and Matthew 2000). Silks are fibrous proteins with unique mechanical properties and have been identified as a suitable scaffold material for in vitro cartilage tissue engineering (Altman *et al.* 2003; Aoki *et al.* 2003; Wang *et al.* 2005; Wang *et al.* 2006).

Growth factors, especially of the transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily can influence the success of chondrogenic induction in vitro and in vivo (Hennig *et al.* 2007). Traditional cartilage inducing growth factors like TGF $\beta$ -1, 2 or 3 have been investigated and are reported as required growth factors for chondrogenesis (Barry *et al.* 2001). Several investigators reported that bone morphogenetic protein 6 (BMP-6), a subgroup of the transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily, plays a role in chondrogenesis (Kameda *et al.* 2000; Sekiya *et al.* 2001). Moreover, the necessity of BMP-6 in chondrogenic induction medium during chondrogenic differentiation of MSCs was demonstrated in several studies (Indrawattana *et al.* 2004; Estes *et al.* 2006; Hennig *et al.* 2007). However, it remains unclear whether a combination of the use of BMP-6 and porous scaffolds might substantially improve cartilage-forming efficacy.

A key question with respect to chondrogenesis in the above studies is how the choice of cell source affects chondrogenic outcomes in 3D systems. Therefore, differences in cell responses on two types of biomaterial scaffolds was assessed in the present work, in order to provide an initial basis for comparative outcomes toward chondrogenesis. This was intended as a feasibility study to demonstrate the success of different cell sources in terms of chondrogenesis in 3D platform. Therefore, cells were collected from young, healthy donors without age or disease dependence. Chondrocytes, ESCs, ESC derived-MSCs and MSCs derived from bone marrow and ASCs derived from adipose tissue were compared in chitosan and silk 3D porous scaffolds.

## 2. Materials and Methods

### 2.1. Preparation of chitosan scaffolds

Chitosan solutions with a concentration of 2% (w/v) were prepared by dissolution of chitosan flakes (deacetylation degree (DD) of a minimum of 85%, Aldrich, Germany) in 0.2 M acetic acid. Porous chitosan scaffolds were prepared by freeze-drying as described in our previous study (Tıg̃lı *et al.* 2007). In brief, chitosan solutions were poured into 24-well

tissue-culture polystyrene dishes (TCPS, TPP Switzerland), with a diameter of 15 mm, to a depth of approximately 5 mm and frozen at  $-20^{\circ}\text{C}$  for 24 h. The samples were transferred into a freeze-drier (Christ, Germany) and lyophilized at  $-80^{\circ}\text{C}$  for 4 days to ensure that they were completely dried. Freshly lyophilized scaffolds were rehydrated with 96% (v/v) ethanol for 1 h then in 70% (v/v) ethanol overnight in order to stabilize the structure. The pore size of the resultant scaffolds was  $\sim 100\ \mu\text{m}$  with an interconnected morphology based on scanning electron microscopy (SEM) (JSM-840A, Japan) (Tıǧlı *et al.* 2007). The scaffolds were cut into discs (8 mm in diameter and 2 mm in thickness).

## 2.2. Preparation of aqueous-derived silk fibroin scaffolds

3-D aqueous-derived silk fibroin scaffolds were prepared according to the procedure described in our previous study (Kim *et al.* 2005). Briefly, a 6% (w/v) silk fibroin aqueous solution was prepared from *Bombyx mori* silkworm cocoons. The cocoons were extracted in a 0.02M  $\text{Na}_2\text{CO}_3$  solution, dissolved in a 9.3M LiBr solution and subsequently dialyzed against distilled water. To form the scaffolds, four grams of granular NaCl particles (600–700  $\mu\text{m}$ ) were added to 2 ml of a 6 (w/v) % silk fibroin solution in Teflon cylinder containers and sit at room temperature for 24 h. The containers were immersed in water to extract the salt from the porous scaffolds for 2 days. The pore size of the resultant scaffolds was  $\sim 550\pm 30\ \mu\text{m}$ , based on analysis with a LEO Gemini 982 field emission gun SEM (Kim *et al.* 2005). The scaffolds were cut into discs (8 mm in diameter and 2 mm in thickness) and dried in a fume hood.

## 2.3. Incorporation of BMP-6 into chitosan and silk fibroin scaffolds

A minimal concentration of BMP-6 shown to enhance chondrogenesis in pellet cultures (Sekiya *et al.* 2001) was selected for this study. Incorporation of BMP-6 (R&D Systems, USA) into the chitosan and silk scaffolds was performed by an embedding technique with  $2\ \mu\text{g}\cdot\text{ml}^{-1}$  BMP-6 solution prepared by dissolving BMP-6 in Dulbecco's Phosphate Buffered Saline (DPBS, without  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$ , Invitrogen). In brief, 50  $\mu\text{l}$  of BMP-6 solution was added drop by drop with a micropipette into the scaffolds and then immediately freeze-dried at  $-80^{\circ}\text{C}$  for 1 day. The amounts of loaded BMP-6 in the scaffolds were 100 ng per dry scaffold by depending upon the concentration of embedding solution. Actual final concentrations of loaded BMP-6 in chitosan and silk scaffolds were measured by RayBio® Human BMP-6 ELISA Kit (Ray Biotech, Inc., USA) following the supplier's instructions.

## 2.4. Cells

Adult primary human chondrocytes (a generous gift from Ivan Martin, Switzerland), P2-P4 were used for this study. Briefly, specimens of healthy articular cartilage tissue were harvested postmortem from full-thickness biopsy specimens of the femoral condyle of a 42 year old individual regarding to the following procedures (Francioli *et al.* 2007). Articular chondrocytes were isolated by 22 hours of incubation at  $37^{\circ}\text{C}$  in 0.15% type II collagenase and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 4.5 mg/ml D-glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.29 mg/ml L-glutamate.

ESC derived mesenchymal stem cells (MSCs) were obtained from two sources. These are denoted as ED1 MSCs and ED2 MSCs, which are from Massachusetts General Hospital (Boston, USA) and WiCell Research Institute (Wisconsin, USA), respectively. ED1 MSCs were prepared by the following protocol. Briefly, human ESCs (h9 cell line, P39) were grown on mouse embryonic fibroblast (MEFs) monolayer cultures and then switched to a differentiation medium (IMDM, 15% FBS) for 10 days. Cells were trypsinized and replated on MSC medium for a few extra days to enrich for MSC-like cells. After sorting for CD73+

cells, these cells were expanded on MSC medium and used for further experiments. The ED2 MSCs were prepared by the following differentiation protocol. Human ESCs (H9 (L), P36) were cultured using WiCell protocols and conditions conducive for the formation of embryoid bodies. The embryoid bodies were generated and cultured in IMDM medium with 10% FBS for 9–11 days. Embryoid bodies were then trypsinized and disaggregated into single cells that were cultured for 7 days in mesenchyme-specific medium (MesenCult™ Basal Medium, Stem Cell Tech., Vancouver, BC) to form mesodermal cells. This cell population for further cultured in mesenchyme-specific medium with predetermined serum (pretested FBS) to form a substantially homogenous population of mesenchymal stem cells. Then they were trypsinized and used for further experiments.

hASCs were obtained from a 30 year old female donor abdomen lipoaspirate (Pennington Biomedical Research Center, Baton Rouge, USA). The hASCs were expanded from collagenase digested stromal vascular fraction cells in Stromal Medium consisting of Dulbecco's modified eagle medium (DMEM)/F12 Ham's medium, 10% FBS, 100 U/ml Penicillin, 100 mg/ml streptomycin and 0.5 µg/ml fungizone. Cells were seeded at a density of 5,000 cells per sq cm and harvested at a ~80–90% confluency with each passage. Total bone marrow aspirate from a healthy, non-smoking 25 year old male donor was obtained from Cambrex (East Rutherford, NJ, USA) and stem cells were freshly isolated. Whole bone marrow cells were plated at a density of 200,000 cells/cm<sup>2</sup> diluted in expansion medium in 175 cm<sup>2</sup> flasks (Corning, Corning, NY, USA). Expansion media consisted of DMEM, 10% FBS, 0.1 mM non-essential amino acids, 1 ng/ml bFGF, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.5 µg/ml fungizone (Gibco, Carlsbad, CA, USA). Each flask contained a final volume of 35 ml, which was rocked daily to allow hematopoietic cells to remain in suspension and the stromal cells to adhere to the flask. The adherent cells were allowed to reach 80% confluence, after which they were trypsinized, suspended in FBS containing 9% DMSO and stored in liquid nitrogen. Cells were thawed, re-plated, expanded for another passage before freezing again. Second passage cells were re-plated once more in expansion media and used for experiments and defined as bone marrow-derived MSCs (BMSCs). Live cultures of human embryonic stem cells (hESCs) H9, normal female karyotype, were obtained from WiCell Research Institute (Wisconsin, USA) and used under NIH approved protocols. Cells at passage 29 were used for this study.

Unless otherwise stated, all tissue culture components were from Invitrogen, USA. Cultures (except ASCs) were expanded in growth medium containing 90% DMEM, 10% FBS, 1% non-essential amino acids, 1% antibiotic/antimycotic and 1 ng/ml basic fibroblast growth factor (bFGF). ASCs were expanded using 90% DMEM-F12 supplemented with 10% FBS, 1% non-essential amino acids and 1% antibiotic/antimycotic. The cells, maintained at 37°C in a humidified CO<sub>2</sub> (5%) atmosphere, were dissociated with 0.25 % trypsin-EDTA at ~80% confluency before being used for subsequent experiments. hESCs cells were maintained according to the instructions from WiCell Research Institute (Wisconsin, USA). Chondrogenesis was induced in a chondrogenic medium (90% DMEM, 10% FBS, 1% non-essential amino acids, 1% antibiotic/antimycotic supplemented with ITS<sup>+</sup> (10 mg/ml insulin, 5.5 mg/ml transferrin, 5 ng/ml selenium, 0.5 mg/ml bovine serum albumin, 4.7 mg/ml linoleic acid; Sigma), 0.1mM ascorbic acid 2-phosphate (Sigma), 10<sup>-7</sup> M dexamethasone (Sigma) and 10 ng/ml TGF-β1.

## 2.5. Fluorescence Activated Cell Sorting (FACS) analysis

All cell sources were characterized with respect to the expression of surface antigens. The expression of the following four surface antigens: CD105 (Biolegend), CD73, CD90 and CD49a (BD Biosciences), were characterized by FACS analysis. Cells were detached with 0.25 % trypsin-EDTA, pelleted, and resuspended in 300 µl DMEM. Cell suspensions were split into three aliquots (100 µl); a control group without staining, 20 µl anti CD73



conjugated with phycoerythrin (CD73-PE) and 20  $\mu$ l anti CD105 conjugated with allophycocyanine (CD105-APC), 20  $\mu$ l CD49a-PE and 5  $\mu$ l CD90-APC. After addition of antibodies the samples were mixed and incubated for 30 min in dark at 4°C. Cells were washed with 1.5 ml DPBS and centrifuged for 5 min at 1500 rpm, 4°C. The supernatant was removed, the cell pellet was suspended in 1 ml DPBS in polystyrene tubes (Falcon) and subjected to FACS analysis.

## 2.6. Cell culture in chitosan and silk fibroin scaffolds

Cell cultures were conducted in sterile 24-well tissue culture plates (TCP, Falcon). Prior to cell culture experiments 24-well TCP were first coated with parafilm, which were presoaked in 70% ethanol, and then placed under UV light for 30 min for sterilization. Chitosan and silk scaffolds were sterilized by brief treatment with 70% ethanol for 30 min and conditioned with DMEM containing 10% FBS for 1 h before cell seeding. Fifty  $\mu$ l of a cell suspension was seeded into each sample (chitosan, BMP-6 loaded chitosan, silk and BMP-6 loaded silk) and allowed to incubate in a humidified incubator (37°C, 5% CO<sub>2</sub>) for 1 h. Finally, 1 ml of chondrogenic medium was added in order to maintain 5  $\times$  10<sup>4</sup> cells/ml inoculation density for each scaffold. The medium was replenished twice a week and all cultures were terminated at day 28.

## 2.7. Cell metabolic activities in scaffolds

Cells in scaffolds were quantitatively assessed with 3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide (MTT, Sigma) formazan each week during the culture period. At selected time intervals, culture medium was aspirated and washed with 600  $\mu$ l prewarmed DPBS. Then 600  $\mu$ l of prewarmed culture medium supplemented with 60  $\mu$ l MTT solution (2.5 mg/ml MTT dissolved in DPBS) was added to each sample and incubated at 37°C for 3 h. After incubation the medium was removed from each well and scaffolds were transferred to another 24-well Petri dish. Then 400  $\mu$ l of 0.04 M HCl in isopropanol solution was added to each well to dissolve the formazan crystals. The resulting solution with crystal violet color was removed and centrifuged at 13,000 rpm for 2 min and 200  $\mu$ l supernatant was used to measure optical density spectrophotometrically at 570 nm using a microplate reader (VERSAmax, Molecular Devices, CA, USA). MTT assay was also applied to the scaffolds without cells as controls and the data was subtracted from the measured values.

## 2.8. Total RNA extraction, cDNA synthesis, and real-time RT-PCR analysis

Total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the supplier's instructions. Briefly, scaffolds seeded with cells were washed with PBS, disrupted and lysed with the supplied buffer (Qiagen). A QIAshredder spin column was used to homogenize the lysate and ethanol was added before transfer to an RNeasy spin column. The final elute was stored at -80°C. Extracted total RNA was used to synthesize cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems) following the supplier's instructions. The cDNA product was stored at -20°C. Real time RT-PCR reactions were conducted and monitored with a Mx3000P (Stratagene®). TaqMan® Gene Expression Assay kits (Applied Biosystems) were used to determine transcript levels of cartilage-related ECM genes including collagen type-II (Col-II), aggrecan (AGC), Sox9, collagen type-X (Col-X) and collagen type-I (Col-I). Five microliters of cDNA from each sample was mixed with 2.5  $\mu$ l of the TaqMan® Gene Expression Assay kit, 17.5  $\mu$ l of RNase/DNase free water, and 25  $\mu$ l of 2  $\times$  TaqMan® Universal PCR Master Mix (Applied Biosystems). The total reaction volume was 50  $\mu$ l. Eukaryotic 18s rRNA endogenous control (Applied Biosystems) was used as a housekeeping/reference transcript. All cDNA samples were analyzed for the transcript of interest and the housekeeping gene in independent reactions. Data were analyzed by MxPro-Mx3000P version 4.0 software supplied by the vendor (Stratagene®). The Ct value for each sample was defined as the cycle number at

which the fluorescence intensity reached a certain threshold where amplification of each target gene was within the linear region of the reaction amplification curve. Relative expression level for each gene of interest was normalized by the Ct value of housekeeping gene 18s using an identical procedure ( $2^{\Delta Ct}$  formula, Perkin Elmer User Bulletin #2). Each sample was analyzed in triplicate.

## 2.9. Histology and SEM

Scaffolds seeded with cells were harvested at week 4, washed in PBS, and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M DPBS for 1 h before histological analysis. For histological evaluation, sections were deparaffinized, rehydrated through a series of graded alcohols, and stained with Safranin-O as well as fast green. The samples were processed with a BenchMark automated histology staining system (Ventana, Tucson, AZ). Sections were counterstained with hematoxylin. For SEM, the scaffolds were gently washed with PBS and cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M DPBS for 1 h. Then the scaffolds were dehydrated in an ethanol series and rinsed with hexamethyldisilazane.

## 2.10. Statistical analysis

All data are expressed as means  $\pm$  standard deviations of three similar experiments carried out in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) in conjunction with Tukey's post hoc test for multiple comparisons using SPSS version 9.0 software.

## 3. Results

### 3.1. Characterization of cells by FACS analysis

The expression of CD105, CD73, CD90 and CD49a surface antigens was analyzed (Figure 1) for all cell sources (chondrocytes, ASCs, ED1 MSCs, ED2 MSCs, BMSCs and ESCs). FACS analysis results showed that the expression of all surface antigens studied was positive for chondrocytes, ASCs, ED1 MSCs, ED2 MSCs and BMSCs (Figure 1A–E) and negative for the ESCs (Figure 1F). Relative fluorescence intensities of surface markers with respect to control IgGs for each cell source were found to be slightly different from each other. CD105, CD90 and CD 49a showed the highest fluorescence intensities for ASCs (Figure 1B) whereas CD73 has the highest level for ED2 MSCs (Figure 1D) (data not shown).

### 3.2. Metabolic activities of cells in chitosan and silk scaffolds

The metabolic activity of all the cell sources in chitosan and silk scaffolds (with or without BMP-6 modification) was evaluated weekly based on MTT analysis (Figure 2). The metabolic activity of chondrocytes in the chitosan scaffolds was significantly higher than the rest of the cell sources ( $p < 0.001$ ) for the first two weeks of incubation period. By the fourth week, all cell types started to proliferate and reached the metabolic activities of the chondrocytes, with no significant differences ( $p > 0.05$ ) except for the ASCs and ED1 MSCs ( $p < 0.05$ ) (Figure 2A). A similar trend was observed for the chitosan-BMP6 scaffolds (Figure 2B). However, significant differences were observed between chondrocytes and the rest of cell sources through the 4 weeks of incubation (Figure 2B). Cells showed approximately the same mitogenic activity on chitosan and BMP-6 modified chitosan scaffolds during the first week of incubation ( $p > 0.05$ ) (Figure 2B). After the third week, a significant effect of BMP-6 was seen on the metabolic activity of the BMSCs and ED2 MSCs ( $p < 0.001$ ) (Figure 2B). The metabolic activities of all of the cell types were found to be significantly higher in the silk scaffolds compared to the chitosan scaffolds (Figure 2C–D). The results demonstrated that chondrocytes and ED1 MSCs, with having the highest metabolic activity, expanded

most rapidly in the silk scaffolds (Figure 2C). On the other hand, the BMSCs and ESCs had the lowest metabolic activities and this continued through 4 weeks of incubation (Figure 2C). The cells showed similar metabolic activities without statistical significant differences ( $p>0.05$ ) between the silk and silk-BMP-6 scaffolds (Figure 2C–D).

### 3.3. Expression of cartilage-related ECM genes by cells cultured in chitosan and silk scaffolds

Transcript levels of cartilage-related ECM genes among the various cultures at four weeks incubation was assessed. The baseline control (18S) whose expression of mRNA was found to be consistent between all different cells used. Transcript levels of AGC and Col-II in ED1 MSCs within the silk constructs cultured with were significantly upregulated in comparison to chondrocytes cultured in the silk constructs ( $p<0.001$ , Figure 3A). Moreover, ED1 MSCs and chondrocytes in silk scaffolds expressed the lowest transcript levels of Col-I, confirming chondrogenesis (Figure 3A). However, the level of Col-X (hypertrophic cartilage marker) was significantly higher in ED1 MSCs and ED2 MSCs (Figure 3A). The transcript levels of Col-I and Col-II in ESCs cultured within the silk scaffolds were significantly upregulated ( $p<0.001$ ) and non-detectable, respectively (Fig. 3A). Similar comparative gene expression results were determined by comparisons of all cell types cultured in BMP-6 constructs (Figure 3B). However, non-detectable transcript levels were maintained for all genes in silk scaffolds cultured with ESCs. Levels of Col-I, Col-II, Sox-9, Col-X (for all cell types) and AGC (for ED2 MSCs) were significantly upregulated in the presence of BMP-6 (Figure 3B). Chitosan scaffolds cultured with ED1 MSCs and ASCs had the highest and significant transcript level of AGC (Figure 3C). Col-II and Col-X expression in ED1 MSCs and ED2 MSCs cultured within chitosan scaffolds were upregulated significantly (Figure 3C), whereas low transcript levels of Col-I were determined for these cell sources. However, ESCs expressed the highest levels of Col-I and Col-X and non-detectable transcript levels for AGC, Col-II and Sox-9 (Figure 3C). ASCs, ED1 MSCs and ED2 MSCs cultured in BMP-6-chitosan scaffolds showed significant and high levels of AGC ( $p<0.001$ , Figure 3D). Col-X was upregulated significantly only for ED2 MSCs and the lowest transcript levels of Col-I were maintained for chondrocytes, ASCs and ED1 MSCs (Figure 3D). Moreover, Col-I was downregulated significantly for ASCs in the presence of BMP-6 (Figure 3D).

### 3.4. Histology and SEM

SEMs showed that chondrocytes, ED1 MSCs and ED2 MSCs, formed continuous sheets of cells, filled the interconnected pores of the silk scaffolds by the end of 4 weeks of cultivation both in the absence and the presence of BMP-6 (Figure 4A–F). The morphologies of chondrocytes on silk and BMP-6-silk scaffolds were flat and elongated (Figure 4A,B). On the outer region of silk scaffolds ED1 MSCs and ED2 MSCs were observed to form multilayers where small round shaped cells were embedded in the pores of scaffolds (Figure 4C, E). Moreover, ED1 MSCs on BMP-6-silk scaffolds acquired a spherical morphology, and were distributed homogeneously on the outer zone of scaffold (Figure 4D). Cell morphologies of ED1 MSCs were spherical on the chitosan scaffolds both in the absence or presence of BMP-6 (Figure 5C, D). In contrast, only in the presence of BMP-6, did the chondrocytes and ED2 MSCs acquire rounded morphology (Figure 5B, F).

The histological evaluations for silk and chitosan scaffolds resulted in weak but positive staining for proteoglycans with Safranin O for ED1 MSCs and ED2 MSCs, both in the presence and absence of BMP-6 (Figure 6C–F, I–L). Minimally positive staining and limited ECM deposition were observed for chondrocytes (Figure 6A, B, G, H) and similar observations were found for the other cell sources (data not shown). In the chitosan scaffolds, the ECM deposition was less extensive than in silk scaffolds (Figure 6A–F and Figure 6G–L, respectively) and clustered ECM deposition was observed within pores of the



chitosan scaffolds while a continuous layer of ECM deposition was achieved for the silk scaffolds.

#### 4. Discussion

Successful cartilage tissue engineering requires three important elements, a safe and easily accessible cell source, a controllable cultivation environment with chondrogenic medium and a biocompatible and degradable 3-D scaffold with favorable structural features for cell attachment, cell metabolic activities and chondrogenesis. Chondrocytes and BMSCs have been shown to be reliable cell sources for chondrogenesis with silk scaffolds (Wang *et al.* 2005; Hofmann *et al.* 2006; Wang *et al.* 2006) under appropriate culture conditions. Chondrocytes, BMSCs and ASCs cultured in chitosan scaffolds (Lahiji *et al.* 2000; Sechriest *et al.* 2000; Malafaya *et al.* 2006; Guo *et al.* 2007) have been investigated as cell sources for cartilage tissue engineering. The present study was motivated by the need to determine suitable cell source options for the *in vitro* formation of cartilage. Data were collected for human chondrocyte, MSCs (derived from bone marrow, adipose), two types of embryonic stem cell-derived MSCs and embryonic stem cell cultivation on two types of porous scaffolds, silk fibroin and chitosan. BMP-6 was also assessed in support of chondrogenesis. The scaffolds used in the present study are all biocompatible and biodegradable, and have highly porous structures with large, interconnected pores (Kim *et al.* 2005; Tıǧlı *et al.* 2007). Traditional chondrogenic inducers like TGF $\beta$ , insulin and dexamethasone, in combination with BMP6 was reported to support chondrogenic differentiation (Indrawattana *et al.* 2004; Estes *et al.* 2006; Hennig *et al.* 2007). Effective amounts of BMP-6 were 500 ng/ml for  $(1.5-2)\times 10^5$  cells (Sekiya *et al.* 2001; Estes *et al.* 2006).

The results in the present study suggest that selecting the appropriate cell source was important in terms of cartilage outcomes, since metabolic activity and differentiation of the cell sources demonstrated significant variation. Cell characterization was first assessed by FACS analysis and negative response was found for all of the antibodies for the ESCs since they are undifferentiated (Figure 1F). Positive expression of CD73, CD105 and CD90 was observed for all MSCs and chondrocytes (Figure 1). These results confirm the minimum criteria for defining multipotent MSCs (Dominici *et al.* 2006). The positive expression of CD49a, is known as a measure of retention of differentiation potential for fibroblast colony-forming unit (CFU-F) generated in MSCs (Deschaseaux *et al.* 2003). This feature was detected for all of the MSCs and chondrocyte precursors.

The metabolic activity ability of the cells in the chitosan scaffolds was found to be lower when compared to the silk scaffolds. This result may be due to the smaller pores in the chitosan scaffolds (~100  $\mu$ m) in conjunction with diffusion limitations. This conclusion is likely, as differences have also been shown in silk scaffolds with different sized pores (Karageorgiou and Kaplan 2005). The results showed that the metabolic activity of all the cell sources was not affected by the presence of BMP-6 for both the chitosan and silk scaffolds. A similar phenomenon has been observed from our previous studies with chitosan scaffolds cultured with ATDC5 cells (data not shown, unpublished). MTT studies showed that chondrocytes, BMSCs and ESCs had high cell metabolic activity capacity in chitosan scaffolds, while chondrocytes, ED1 MSCs and ED2 MSCs showed this type of metabolic activities in the silk scaffolds. Moreover, the metabolic activity of chondrocytes in both silk and chitosan scaffolds was significantly higher than the stem cells, in agreement with our previous results (Wang *et al.* 2006).

The differentiation of all of the cell sources in the 3-D silk fibroin and chitosan scaffolds was evident by the upregulation of mRNAs for cartilage-related genes (AGC, Col-II, Col-X and Sox-9). The upregulated AGC, Col-II and Sox-9 genes in embryonic stem cells and

MSCs suggest improved differential potential for these cell sources compared to chondrocytes. However, it should be noted that cell density was critical for the redifferentiation of culture-expanded human chondrocytes in the 3-D like in aqueous-derived silk fibroin scaffolds (Wang *et al.* 2006). On the other hand, the results reflect the inherent tendency for MSCs to express Col-I mRNA. Similar results were observed from our previous study suggesting BMSCs tend to express Col-I, which can be regulated by environmental factors like culture format (2D vs 3D) and biochemical factors (TGF $\beta$ 1, 2, 3) (Wang *et al.* 2005). In addition, previous studies reported that MSCs could be further differentiated to the hypertrophic state and low-density seeded chondrocytes resulted in the expression of Col-X (Barry *et al.* 2001; Wang *et al.* 2005). In this study, Col-X, a hypertrophic cartilage-specific gene, was upregulated for all cell types. However, it is noted that the transcript level of Col-X was significantly higher for MSCs derived from embryonic stem cells while BMSCs expressed the lowest level of Col-X. The undetectable Col II for AMSCs and BMSCs seed in chitosan scaffolds may be due to incomplete differentiation of cells, since these cells in BMP-6 chitosan scaffolds did express Col II. These results concluded the importance of BMP-6 for cell differentiation in chitosan scaffolds.

Although all these results represent the general view of chondrogenic differentiation for both chitosan and silk scaffolds, it should be noted that the differentiation behavior of cell types varied significantly among scaffold types. Moreover, transcript levels were affected by the presence or absence of BMP-6. Consequently, for the silk scaffolds with or without BMP-6, ED1 MSCs and BMSCs, for chitosan scaffolds ED1 MSCs and for chitosan-BMP6 scaffolds ED1 MSCs and ASCs were found to more closely resemble the chondrogenic phenotype. The most extensive cartilage specific ECM depositions for both silk and chitosan scaffolds were achieved for ED1 MSCs and ED2 MSCs. These results indicated the differentiation capacity of these cell sources which was consistent with the PCR results. However, it should be noted that weak and nonhomogeneous staining was observed for all samples, suggesting incomplete chondrogenesis. This was likely due to the relatively short culture period of four weeks. Freed *et al.* successfully reported chondrocytes, cultured on PGA scaffolds in rotating bioreactors, formed cartilaginous tissues after 40 days and mentioned time-dependent chondrogenesis (Freed *et al.* 1998). However, since our goal in the present study was to assess chondrogenic potential from the various cell sources, four weeks was sufficient to elucidate differences based on the transcript levels and related cell data.

The morphology of cells seeded on the silk and chitosan scaffolds was observed after four weeks of incubation. In previous studies, a spherical cell morphology was observed in 3D cultures of MSCs (Williams *et al.* 2003) and chondrocytes (Benya and Shaffer 1982), which related to the synthesis of ECM components representative of cartilage (von der Mark *et al.* 1977). However, it has been shown that rounded chondrocytes can synthesize type I collagen and spread chondrocytes can express type II collagen (von der Mark *et al.* 1977; Mallein-Gerin *et al.* 1990), suggesting that cell shape may not be critical in influencing chondrocyte differentiation. In this study, we demonstrated that ED1 MSCs acquired rounded morphologies on the silk-BMP-6, chitosan and chitosan-BMP-6 scaffolds. Moreover ED2 MSCs and chondrocytes on the chitosan-BMP-6 scaffolds retained chondrocytic phenotype. In addition, ED1 MSCs and ED2 MSCs formed small round shaped cells within the pores of the silk scaffolds, suggesting a more favorable environment for the retention of the rounded morphology (Barry *et al.* 2004).

A key point, which should be considered in the present work is the variation even in some of the basic characteristics among the cell sources studied, including age and sex of the donors, passage number of isolated cells, cell seeding levels, and differentiation protocols. The chondrocytes (P2–P4) were isolated from a 42 year old male patient, the ASCs (P2) and BMSCs (P3) were obtained from a 30 year old female and 25 year old male donor,

respectively, and the passage number of hESCs (normal female karyotype), ED1 MSCs and ED2 MSCs, were P29, P6 and P4, respectively. ED1 MSCs were differentiated using a 2D system while ED2 MSCs were obtained after differentiation in embryoid body, which was previously suggested to enhanced osteogenesis in vitro (Karp *et al.* 2006). Although our results represent a comparative evaluation of different cell types in 3D scaffolds in terms of chondrogenesis, the variables listed above should be considered when making choices for cell sources. Thus, further study would be required to fully understand the impact of age of donor, expansion protocol, sex of donor and related variables on chondrogenic outcomes for each selective cell source studied here.

## 5. Conclusions

In the present study, a variety of cell sources was compared in 3D culture for chondrogenic outcomes. The cell sources included chondrocytes, MSCs (derived from bone marrow, adipose tissue and human embryonic stem cells) and hESCs, and they were cultivated on 3D porous scaffolds consisting of silk and chitosan with/without BMP-6. The metabolic activity of cells was not significantly affected by the presence of BMP-6, and was found to be higher in the silk scaffolds compared to the chitosan scaffolds. Successful differentiation as well as metabolic activity of MSCs in both silk and chitosan scaffolds was achieved compared to the chondrocytes and embryonic stem cells. However, differences in differentiation of all of the cell sources of MSCs were determined, based on genotypic and phenotypic assessments of markers for cartilage tissue. The success of supporting chondrogenesis for ED1 MSCs was evident in both the silk and chitosan scaffolds. The presence of BMP-6 induced the upregulation of cartilage specific markers and extended the area of ECM deposition, and also regulated the preservation of chondrocytic morphology of ED1 MSCs in the silk scaffolds. Consequently, BMP-6 modified silk scaffolds cultured with ED1 MSCs are suggested as the most promising system to pursue for cartilage regeneration. However, chondrogenesis was incomplete and was not homogeneous throughout scaffold, which suggests the need for further studies based on culture conditions, such as dynamic cultivation. Further, while attempts were made to utilize comparative cell numbers for the comparisons, variables in passage numbers must also be considered in future work. Further, comparisons between the two main scaffold types, silk and chitosan, must also await further clarification of the influence of pore size on the outcomes reported here. Nonetheless, comparisons among a scaffold type are instructive for selection of cell source for cartilage studies.

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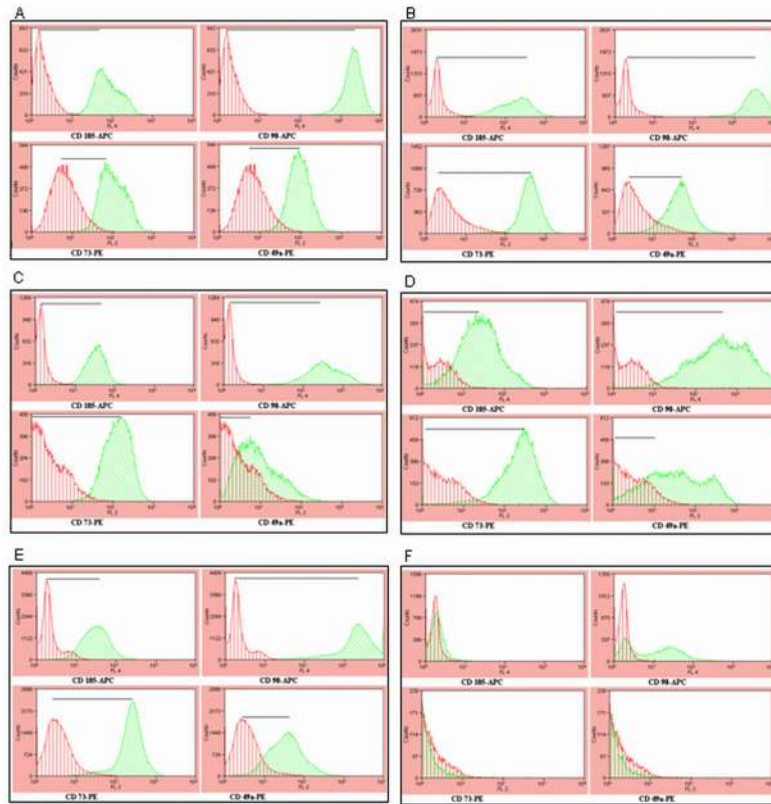
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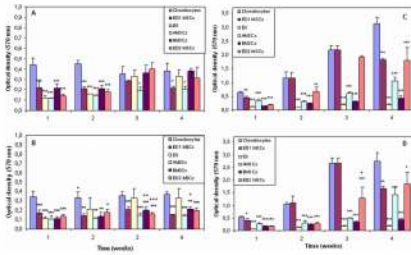


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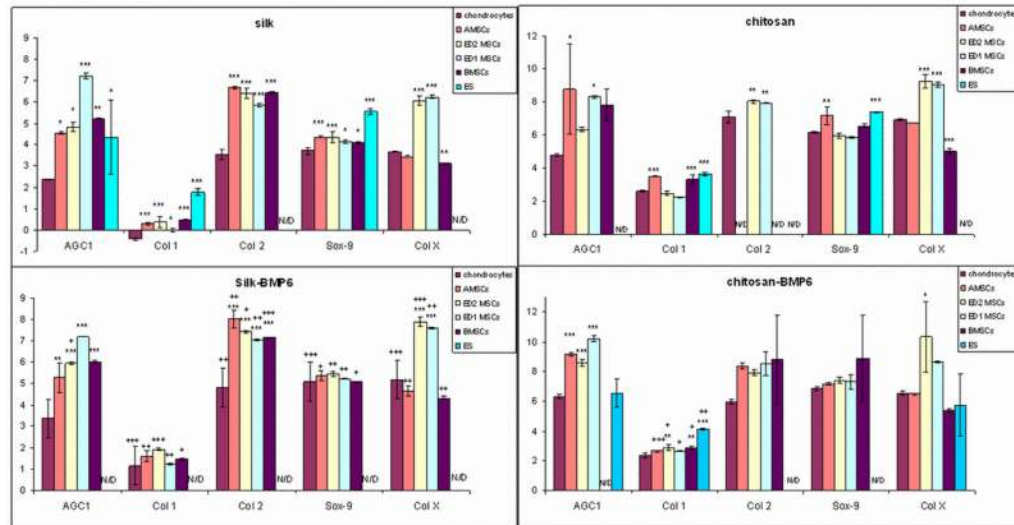
**Figure 1.** Characterization of the different sources of cells for surface markers: CD105-APC, CD73-PE, CD90-APC and CD49a-PE. (A) chondrocytes, (B) ASCs, (C) ED1 MSCs, (D) ED2 MSCs, (E) BMSCs, (F) ESCs. Red and green histograms showed control and surface marker IgGs, respectively. The bars on the peak levels of histograms are a measure of positive expression of surface markers.



**Figure 2.**

Figure 2A–B. MTT results showing the metabolic activities of cells in chitosan (A), BMP-6-chitosan scaffolds (B). Statistical differences between cell sources (control group is chondrocytes,  $n=3$ , \*  $p<0.05$ ; \*\*  $p<0.01$ ; \*\*\*  $p<0.001$ ). Statistical differences between chitosan and BMP-6-chitosan scaffolds for all cell sources (control group is chitosan,  $n=3$ , +  $p<0.05$ ; ++  $p<0.01$ ; +++  $p<0.001$ ).

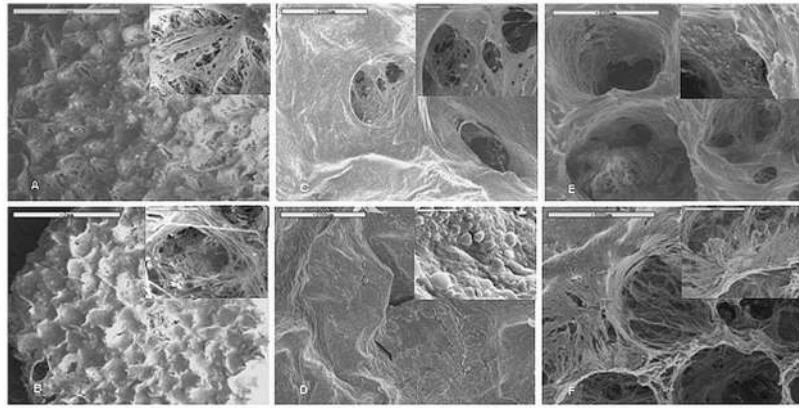
Figure 2C–D. Cell metabolic activities in silk scaffolds: (C), BMP-6-silk scaffolds (D). Statistical differences between cell sources (control group is chondrocytes,  $n=3$ , \*  $p<0.05$ ; \*\*  $p<0.01$ ; \*\*\*  $p<0.001$ ). Statistical differences between silk and BMP-6-silk scaffolds for all types of cell sources (control group is silk,  $n=3$ , +  $p<0.05$ ; ++  $p<0.01$ ; +++  $p<0.001$ ).



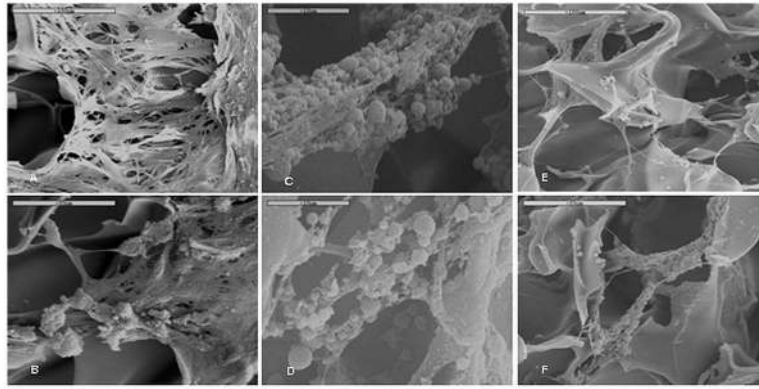
**Figure 3.**

Figure 3A–B. Log-fold transcript levels of cells in silk (A) and silk-BMP-6 (B) scaffolds. All samples were collected and normalized against 18S at week four. Statistical differences between cell sources for silk and silk-BMP-6 (control group is chondrocytes, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). Statistical differences between silk and silk-BMP-6 (silk is the control group, +  $p < 0.05$ , ++  $p < 0.01$ , +++  $p < 0.001$ ). N/D (non detectable).  
 Figure 3C–D. Log-fold transcript levels of cells in chitosan (C) and chitosan-BMP-6 (D) scaffolds. All samples were collected and normalized to 18S at week four. Statistical differences between cell sources for chitosan and chitosan-BMP-6 (control group is chondrocytes, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). Statistical differences between chitosan and chitosan-BMP-6 (chitosan is control group, +  $p < 0.05$ , ++  $p < 0.01$ , +++  $p < 0.001$ ). N/D (non detectable).

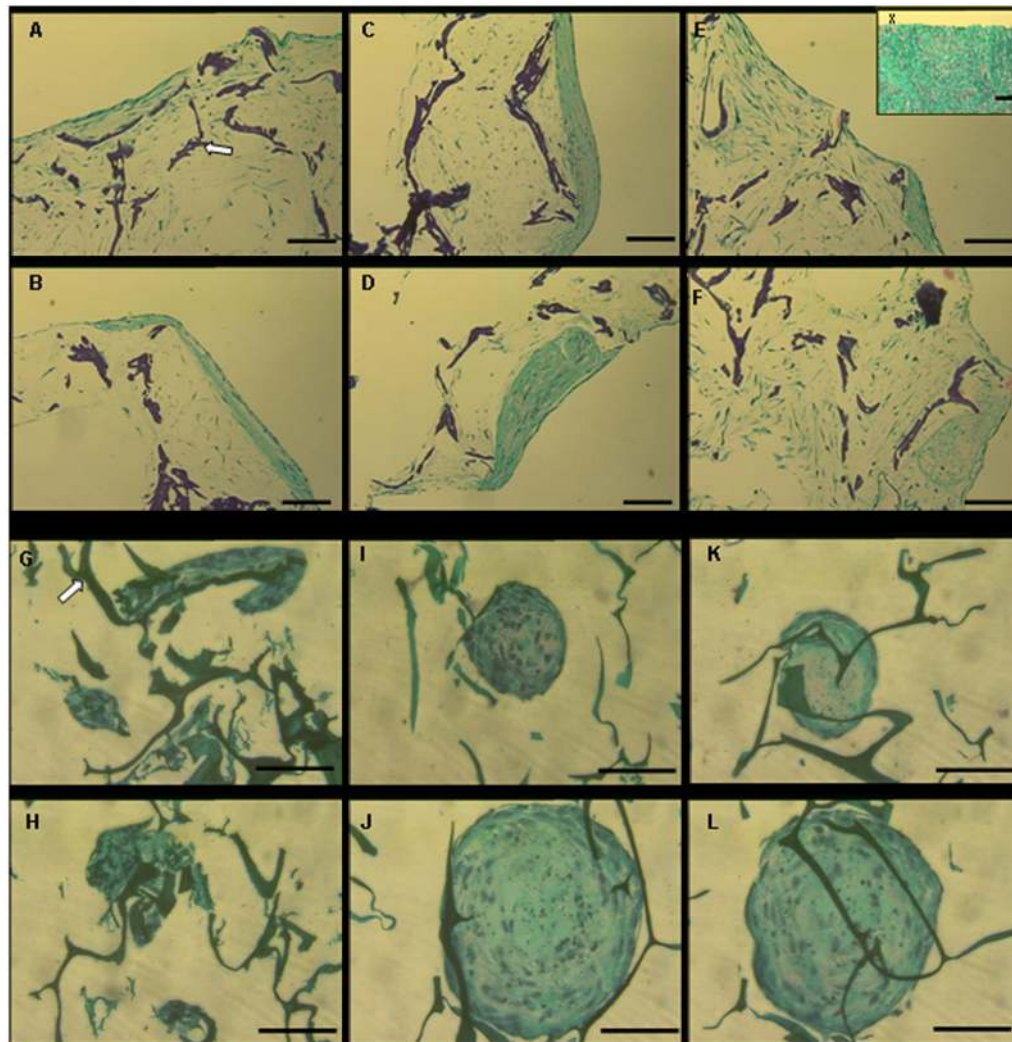




**Figure 4.** SEM images of cells cultured for four weeks on silk and BMP-6-silk scaffolds. Chondrocytes (A), ED1 MSCs (C) and ED2 MSCs (E), on silk scaffolds. Chondrocytes (B), ED1 MSCs (D) and ED2 MSCs (F), on BMP6-silk scaffolds.



**Figure 5.** SEM images of cells cultured for four weeks on chitosan and BMP-6-chitosan scaffolds. Chondrocytes (A), ED1 MSCs (C) and ED2 MSCs (E), on chitosan scaffolds. Chondrocytes (B), ED1 MSCs (D) and ED2 MSCs (F), on BMP6-chitosan scaffolds.



**Figure 6.**

Histological evaluation of cells cultured on silk, BMP-6-silk, chitosan and BMP-6-chitosan scaffolds at four weeks. Staining of native cartilage with Safranin O is demonstrated in Figure X (bar is 200  $\mu\text{m}$ ,  $\times 10$  magnification) used as control. (A, C, E) are silk scaffolds and (B, D, F) are silk-BMP-6 scaffolds with  $\times 10$  magnification. (G, I, K) are chitosan and (H, J, L) are chitosan-BMP-6 scaffold with  $\times 32$  magnification. The scale bars for silk and chitosan scaffolds are 200 and 100  $\mu\text{m}$ , respectively. The cell sources are chondrocytes (A, B, G, H), ED1 MSCs (C, D, I, J) and ED2 MSCs (E, F, K, L). Arrows ( $\Rightarrow$ ) indicate the frame of scaffolds.