JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2009; **3**: 348–360.
Published online 20 April 2009 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/term.169

RESEARCH ARTICLE

Comparative chondrogenesis of human cell sources in 3D scaffolds

R. Seda Tığlı[1,2], Sourabh Ghosh[1], Michael M. Laha[1], Nirupama K. Shevde[3], Laurence Daheron[4], Jeffrey Gimble[5], Menemşe Gümüşderelioğlu[2] and David L. Kaplan[1]*
[1]*Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA*
[2]*Hacettepe University, Chemical Engineering Department, 06800, Beytepe-Ankara, Turkey*
[3]*WiCell Research Institute, Madison, WI 53707-7365, USA*
[4]*Massachusetts General Hospital, Center for Regenerative Medicine and Technology, 185 Cambridge Street, Boston, MA 02114, USA*
[5]*Pennington Biomedical Research Center, 6400 Perkins Road, 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 (BMP6), 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 microscopical analyses. After 4 weeks of cultivation, embryonic stem cells-derived MSCs were promising for chondrogenesis, particularly in the silk scaffolds with BMP6. The results suggest that cell source differences are important to consider with regard to chondrogenic outcomes, and among the variables addressed here the human embryonic stem cells-derived MSCs were the preferred cell source. Copyright © 2009 John Wiley & Sons, Ltd.

Received 5 December 2008; Revised 27 February 2009; Accepted 9 March 2009

Keywords **chondrocytes; stem cells; silk; chitosan; chrondrogenesis; scaffold**

1. Introduction

Severe damage to cartilage tissue caused by developmental abnormalities, trauma or ageing-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 extracellular matrix (ECM) formation (Langer and Vacanti, 1993).

*Correspondence to: David L. Kaplan, Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA. E-mail: David.Kaplan@tufts.edu

Chrondrocytes 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 multilineage 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, 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 in which 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 three-dimensional (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 BMSCs 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(ε -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, there may be 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 (Athanasiou *et al*., 1996; Suh, 1998). Naturally occurring polymers as scaffolds offer options for cartilage tissue engineering, due to biocompatibility, biodegradability, low toxicity of degradation by-products 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 such as 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 materials for *in vitro* cartilage tissue engineering (Altman *et al*., 2003; Aoki *et al*., 2003; Wang *et al*., 2005, 2006).

Growth factors, especially of the transforming growth factor beta (TGFβ) superfamily can influence the success of chondrogenic induction *in vitro* and *in vivo* (Hennig *et al*., 2007). Traditional cartilage-inducing growth factors such as TGFβ1, TGFβ2 or TGFβ3 have been investigated and are reported as required growth factors for chondrogenesis (Barry *et al*., 2001). Several investigators have reported that bone morphogenetic protein 6 (BMP6), a subgroup of TGFβ superfamily, plays a role in chondrogenesis (Kameda *et al*., 2000; Sekiya *et al*., 2001). Moreover, the necessity of BMP6 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 BMP6 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,

DOI: 10.1002/term

differences in cell responses on two types of biomaterial scaffolds were assessed in the present study, 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 platforms. Therefore, cells were collected from young, healthy donors without age or disease dependence. Chondrocytes, ESCs, ESC-derived MSCs, 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 (Tığlı *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 °C for 24 h. The samples were transferred into a freeze-dryer (Christ, Germany) and lyophilized at −80 °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 ∼100 μm, with an interconnected morphology based on scanning electron microscopy (SEM; JSM-840A, Japan) (Tığlı *et al.*, 2007). The scaffolds were cut into discs (diameter 8 mm, thickness 2 mm).

2.2. Preparation of aqueous-derived silk fibroin scaffolds

3D 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 0.02 M Na_2CO_3 solution, dissolved in a 9.3 M LiBr solution and subsequently dialysed against distilled water. To form the scaffolds, 4 g granular NaCl particles (600–700 μm) were added to 2 ml 6% w/v silk fibroin solution in Teflon cylinder containers and kept 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 ∼550 ± 30 μm, based on analysis with a LEO Gemini 982 field emission gun SEM (Kim *et al.*, 2005). The scaffolds were cut into discs (diameter 8 mm, thickness 2 mm) and dried in a fume hood.

2.3. Incorporation of BMP6 into chitosan and silk fibroin scaffolds

A minimal concentration of BMP6 shown to enhance chondrogenesis in pellet cultures (Sekiya *et al.*, 2001) was selected for this study. Incorporation of BMP6 (R&D Systems, USA) into the chitosan and silk scaffolds was performed by an embedding technique with 2 μg/ml BMP6 solution prepared by dissolving BMP6 in Dulbecco's phosphate-buffered saline (DPBS, without Ca^{+2} and Mg^{+2}; Invitrogen). In brief, 50 μl BMP6 solution was added drop by drop with a micropipette into the scaffolds and then immediately freeze-dried at −80 °C for 1 day. The amounts of loaded BMP6 in the scaffolds were 100 ng/dry scaffold by depending upon the concentration of embedding solution. Actual final concentrations of loaded BMP6 in chitosan and silk scaffolds were measured by RayBio® Human BMP6 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 post mortem from full-thickness biopsy specimens of the femoral condyle of a 42 year-old individual according to the following procedures (Francioli *et al.*, 2007). Articular chondrocytes were isolated by 22 h incubation at 37 °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 non-essential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 units/ml penicillin, 100 μg/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, MA, 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 disaggreated into single cells that were cultured for 7 days in mesenchyme-specific medium (MesenCult™ Basal Medium, Stem Cell

DOI: 10.1002/term

Technology, 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 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 5000 cells/cm^2 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^2 diluted in expansion medium in 175 cm^2 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 haematopoietic 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 and expanded for another passage before freezing again. Second passage cells were re-plated once more in expansion medium 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 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. 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_2 (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. Chondrogenesis was induced in a chondrogenic medium (90% DMEM, 10% FBS, 1% non-essential amino acids, 1% antibiotic/antimycotic supplemented with ITS^{+1} (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), 0.1 mM ascorbic acid 2-phosphate (Sigma), 10^{-7} M dexamethasone (Sigma) and 10 ng/ml TGFβ1.

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 μl anti-CD105 conjugated with allophycocyanine (CD105–APC), 20 μl CD49a–PE and 5 μl CD90–APC. After addition of antibodies the samples were mixed and incubated for 30 min in the dark at 4 °C. The 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 TCPs were first coated with parafilm, which was 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. 50 μl of a cell suspension was seeded into each sample (chitosan, BMP6-loaded chitosan, silk and BMP6-loaded silk) and allowed to incubate in a humidified incubator (37 °C, 5% CO_2) for 1 h. Finally, 1 ml chondrogenic medium was added in order to maintain 5×10^4 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 μl prewarmed DPBS. Then 600 μl prewarmed culture medium supplemented with 60 μl 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 μl 0.04 M HCl in isopropanol

DOI: 10.1002/term

solution was added to each well to dissolve the formazan crystals. The resulting solution with crystal violet colour was removed and centrifuged at 13 000 rpm for 2 min and 200 µl 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 using an 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*). cDNA (5 µl from each sample) was mixed with 2.5 µl TaqMan® Gene Expression Assay kit, 17.5 µl RNase/DNase-free water and 25 µl 2× TaqMan® Universal PCR Master Mix (Applied Biosystems). The total reaction volume was 50 µl. Eukaryotic *18S* rRNA endogenous control (Applied Biosystems) was used as a housekeeping/reference transcript. All cDNA samples were analysed for the transcript of interest and the housekeeping gene in independent reactions. Data were analysed 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 No. 2). Each sample was analysed 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 and fast green. The samples were processed using a BenchMark automated histology staining system (Ventana, Tucson, AZ, USA). Sections were counterstained with haematoxylin. For SEM, the scaffolds were gently washed with PBS and the 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 ± standard deviations (SDs) 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 analysed (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 had 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 BMP6 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 2 weeks of the 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 the cell sources through the 4 weeks of incubation (Figure 2B). Cells showed approximately the same mitogenic activity on chitosan and BMP6-modified chitosan scaffolds during the first week of incubation

DOI: 10.1002/term

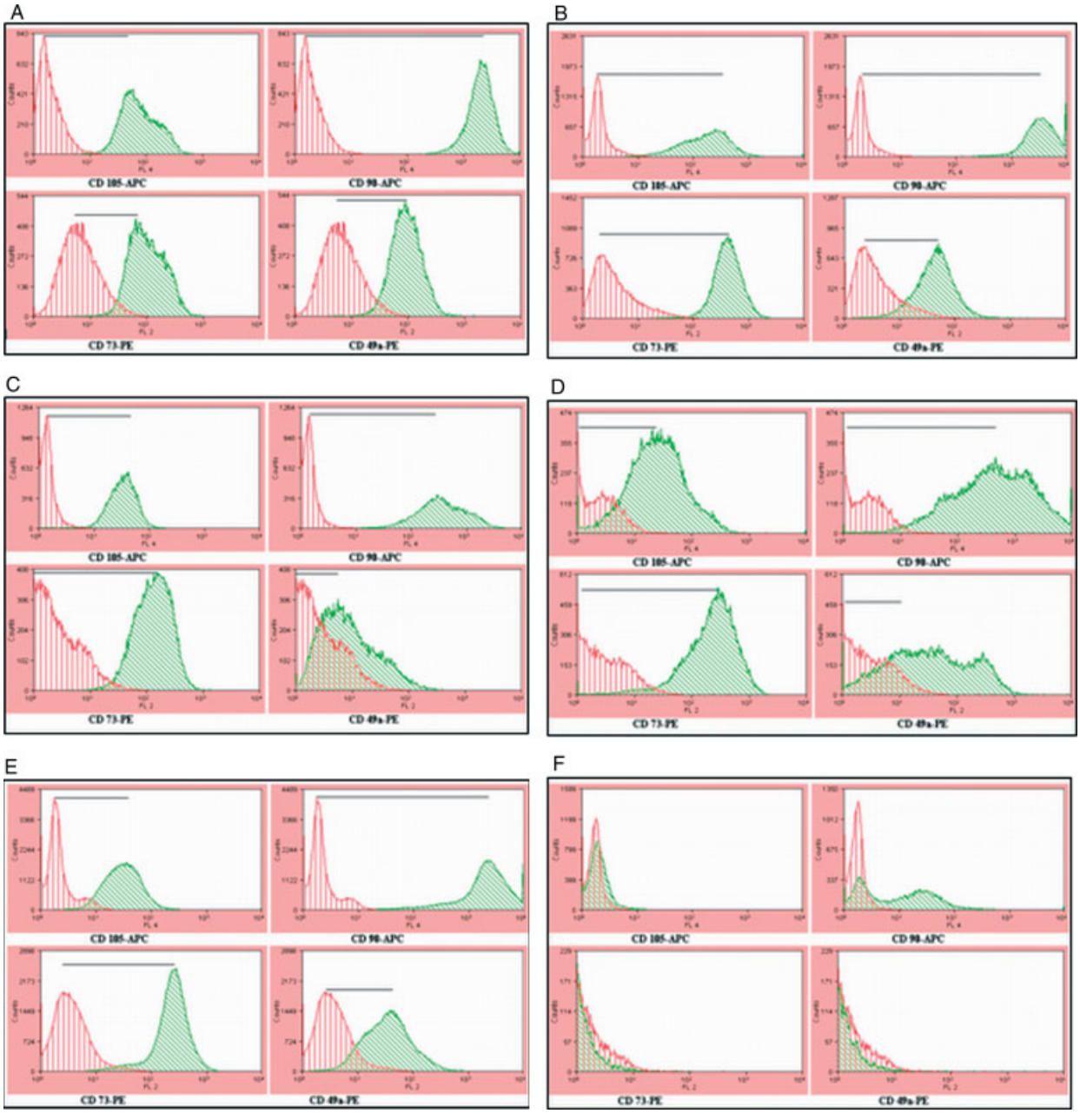

Figure 1. Characterization of the different sources of cells for surface markers: CD105–APC, CD73–PE, CD90–APC and CD49a–PE. (A) chondrocytes, (B) ASCs, (C) ED1 MSCs, (D) ED2 MSCs, (E) BMSCs, (F) ESCs. Red and green histograms showed control and surface marker IgGs, respectively. The bars on the peak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

($p > 0.05$; Figure 2B). After the third week, a significant effect of BMP6 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, 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 statistically significant differences ($p > 0.05$) between the silk and silk–BMP6 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 4 weeks of incubation was assessed. The baseline control was *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,

DOI: 10.1002/term

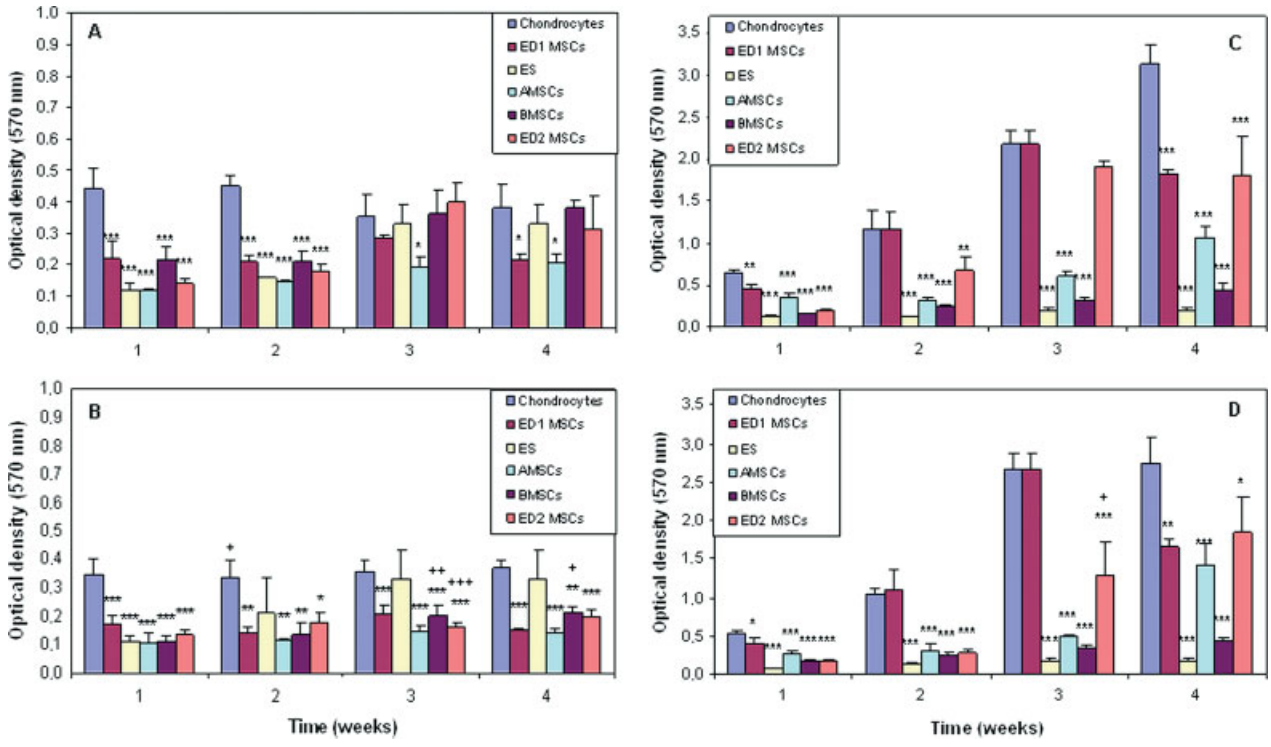

Figure 2. (A, B) MTT results showing the metabolic activities of cells in chitosan (A) and BMP6–chitosan scaffolds (B). Statistical differences between cell sources (control group is chondrocytes, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Statistical differences between chitosan and BMP6–chitosan scaffolds for all cell sources (control group is chitosan, $n = 3$; $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$). (C, D) Cell metabolic activities in silk scaffolds (C) and BMP6–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 BMP6–silk scaffolds for all types of cell sources (control group is silk, $n = 3$; $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$)

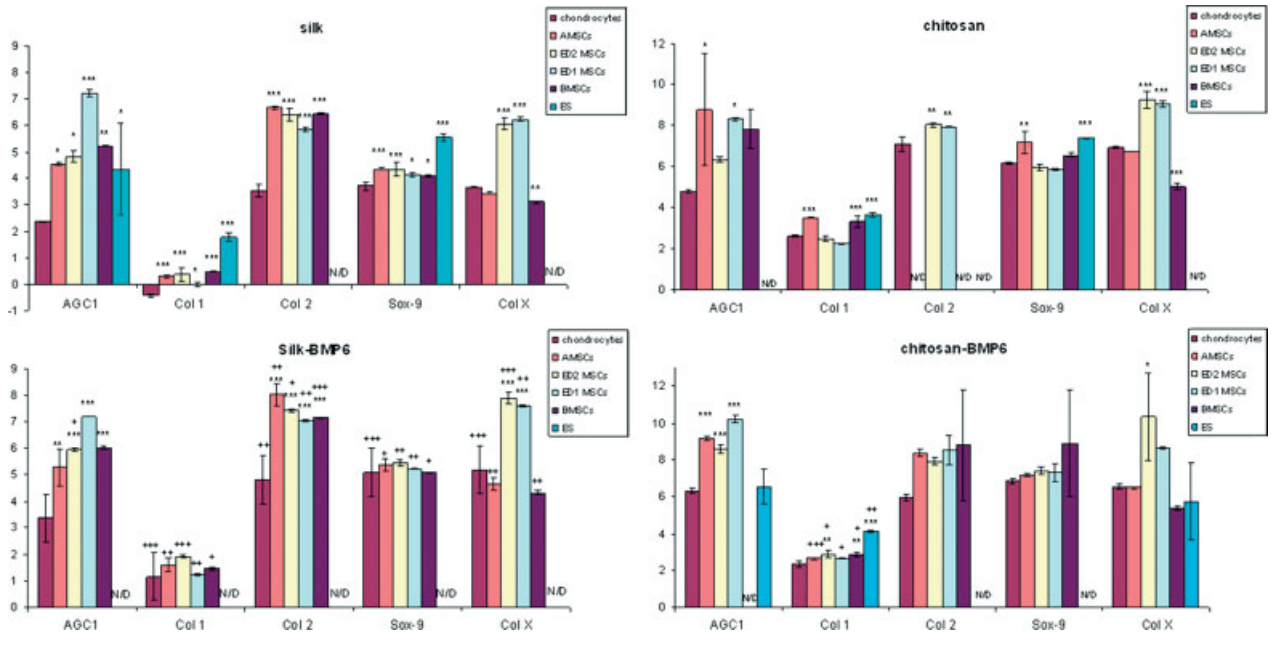

Figure 3. (A, B) Log-fold transcript levels of cells in silk (A) and silk–BMP6 (B) scaffolds. All samples were collected and normalized against *18S* at week 4. Statistical differences between cell sources for silk and silk–BMP6 (control group is chondrocytes; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Statistical differences between silk and silk–BMP6 (silk is the control group; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$). N/D, non-detectable. (C, D) Log-fold transcript levels of cells in chitosan (C) and chitosan–BMP6 (D) scaffolds. All samples were collected and normalized to *18S* at week 4. Statistical differences between cell sources for chitosan and chitosan–BMP6 (control group is chondrocytes; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Statistical differences between chitosan and chitosan–BMP6 (chitosan is control group; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$). N/D, non-detectable

DOI: 10.1002/term

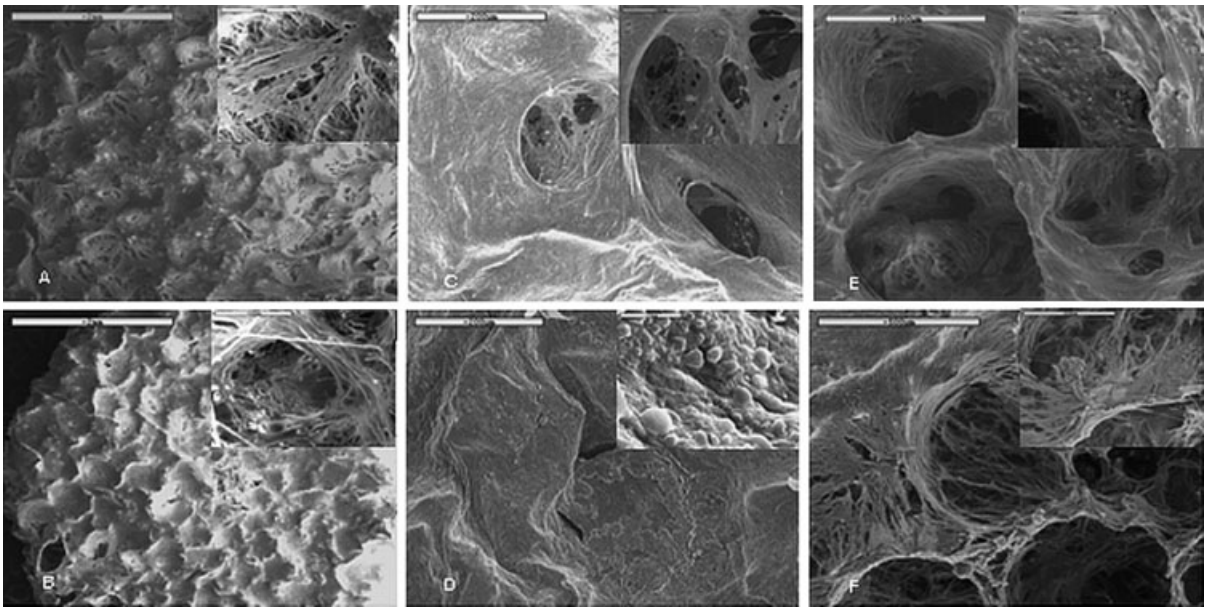

Figure 4. SEM images of cells cultured for 4 weeks on silk and BMP6–silk scaffolds. Chondrocytes (A), ED1 MSCs (C) and ED2 MSCs (E) on silk scaffolds; chondrocytes (B), ED1 MSCs (D) and ED2 MSCs (F) on BMP6–silk scaffolds

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 (Figure 3A). Similar comparative gene expression results were determined by comparisons of all cell types cultured in BMP6 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 BMP6 (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 BMP6–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 BMP6 (Figure 3D).

3.4. Histology and SEM

SEMs showed that chondrocytes, ED1 MSCs and ED2 MSCs formed continuous sheets of cells and filled the interconnected pores of the silk scaffolds by the end of 4 weeks of cultivation, in both the absence and the presence of BMP6 (Figure 4A–F). The morphologies of chondrocytes on silk and BMP6–silk scaffolds were flat and elongated (Figure 4A, B). On the outer regions of the 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 BMP6–silk scaffolds acquired a spherical morphology, and were distributed homogeneously on the outer zones of the scaffolds (Figure 4D). Cell morphologies of ED1 MSCs were spherical on the chitosan scaffolds in both the absence and the presence of BMP6 (Figure 5C, D). In contrast, only in the presence of BMP6 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, in both the presence and the absence of BMP6 (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 the silk scaffolds (Figure 6A–F and Figure 6G–L, respectively) and clustered ECM deposition was observed within the 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

DOI: 10.1002/term

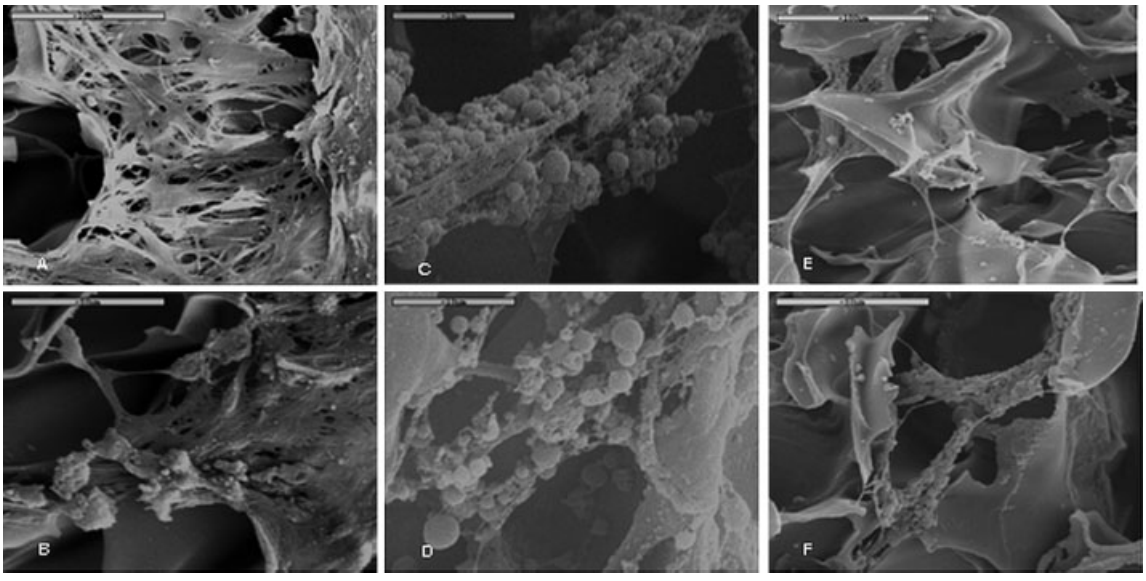

Figure 5. SEM images of cells cultured for 4 weeks on chitosan and BMP6–chitosan scaffolds. Chondrocytes (A), ED1 MSCs (C) and ED2 MSCs (E) on chitosan scaffolds; chondrocytes (B), ED1 MSCs (D) and ED2 MSCs (F) on BMP6–chitosan scaffolds

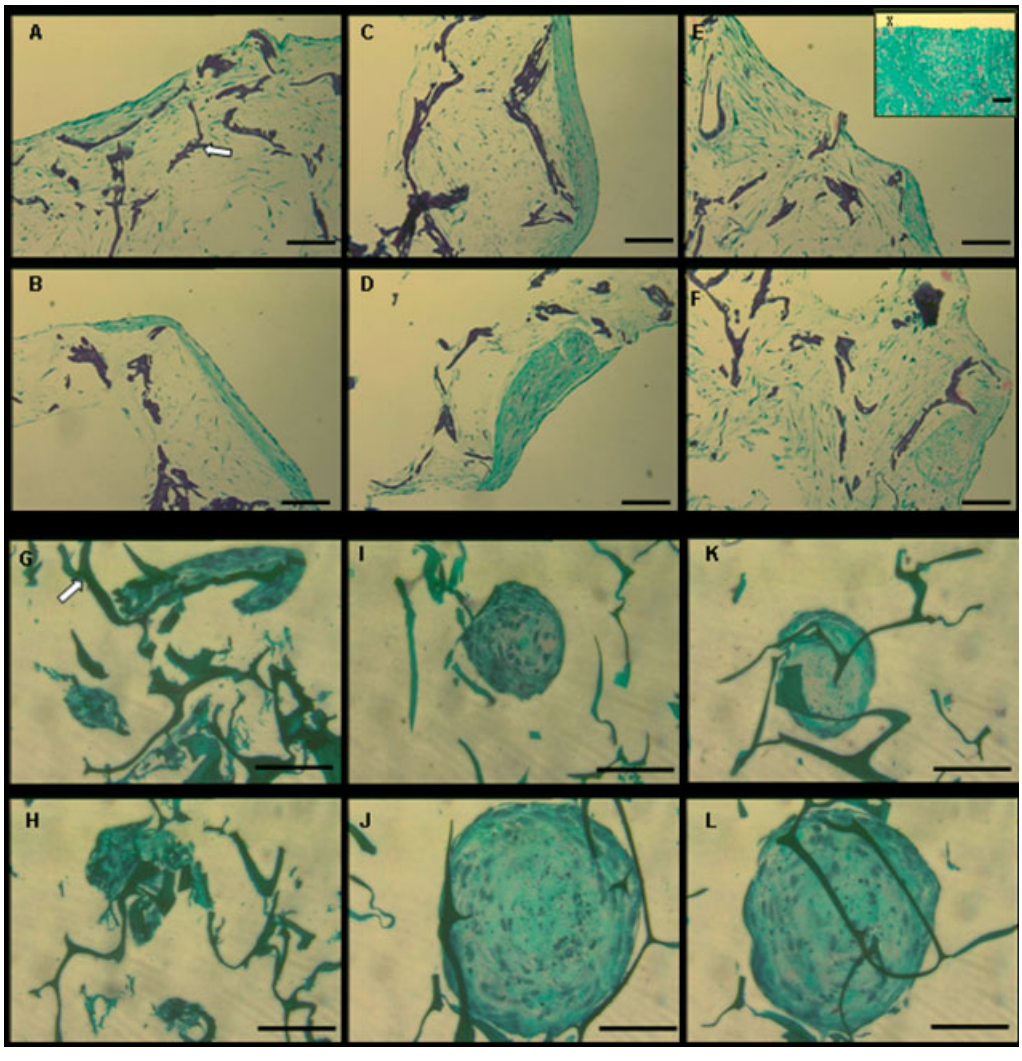

Figure 6. Histological evaluation of cells cultured on silk, BMP6–silk, chitosan and BMP6–chitosan scaffolds at 4 weeks. Staining of native cartilage with safranin O is demonstrated in inset X (bar = 200 μm; magnification ×10) used as control. (A, C, E) Silk scaffolds and (B, D, F) silk–BMP6 scaffolds (magnification ×10). (G, I, K) Chitosan and (H, J, L) chitosan–BMP6 scaffolds (magnification ×32). The scale bars for silk and chitosan scaffolds are 200 and 100 μm, respectively. The cell sources are chondrocytes (A, B, G, H), ED1 MSCs (C, D, I, J) and ED2 MSCs (E, F, K, L). Arrows indicate the frames of scaffolds

DOI: 10.1002/term

source, a controllable cultivation environment with chondrogenic medium and a biocompatible and degradable 3D scaffold with favourable structural features for cell attachment, cell metabolic activites and chondrogenesis. Chondrocytes and BMSCs have been shown to be reliable cell sources for chondrogenesis with silk scaffolds (Wang *et al*., 2005, 2006; Hofmann *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 chondrocytes, 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. BMP6 was also assessed in support of chondrogenesis. The scaffolds used in the present study were all biocompatible and biodegradable and had highly porous structures with large, interconnected pores (Kim *et al*., 2005; Tığlı *et al*., 2007). Traditional chondrogenic inducers such as TGFβ, insulin and dexamethasone, in combination with BMP6, were reported to support chondrogenic differentiation (Indrawattana *et al*., 2004; Estes *et al*., 2006; Hennig *et al*., 2007). Effective amounts of BMP6 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 units (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 ($\sim$100 μm) 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 BMP6 for both the chitosan and the 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 activity 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 3D 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 3D as 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 (TGFβ1, TGFβ2, TGFβ3; 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 seeded in chitosan scaffolds may be due to incomplete differentiation of cells, since these cells in BMP6 chitosan scaffolds did express *Col-II*. These results concluded the importance of BMP6 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 behaviour of cell types varied significantly among scaffold types. Moreover, transcript levels were affected by the presence or absence of BMP6. Consequently, for the silk scaffolds with or without BMP6, 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 non-homogeneous staining was observed for all samples, suggesting incomplete chondrogenesis. This was likely due to the relatively short culture period of 4 weeks. Freed *et al*. (1998) reported that chondrocytes, cultured on PGA scaffolds in rotating bioreactors, formed cartilaginous tissues after 40 days and mentioned time-dependent chondrogenesis. However, since our goal in the present study was to assess chrondrogenic potential

DOI: 10.1002/term

from the various cell sources, 4 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 4 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–BMP6, chitosan and chitosan–BMP6 scaffolds. Moreover, ED2 MSCs and chondrocytes on the chitosan–BMP6 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 favourable environment for the retention of the rounded morphology (Barry *et al*., 2004).

A key point that 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 a 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 an embryoid body, which was previously suggested to enhance 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 chrondrogenic 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 chrondrogenic 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 or without BMP6. The metabolic activity of the cells was not significantly affected by the presence of BMP6, and was found to be higher in the silk scaffolds than in 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 BMP6 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, BMP6-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.

Acknowledgements

R. Seda Tığlı acknowledges a grant provided by the Turkish National Research Council (TÜBİTAK). We thank the NIH P41 Tissue Engineering Resource Center for support of the studies.

References

Afizah H, Yang Z, Hui JHP, *et al*. 2007; A comparison between the chondrogenic potential of human bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs) taken from the same donors. *Tissue Eng* **13**: 659–666.

Altman GH, Diaz F, Jakuba C, *et al*. 2003; Silk-based biomaterials. *Biomaterials* **24**: 401–416.

Aoki H, Tomita N, Morita Y, *et al*. 2003; Culture of chondrocytes in fibroin–hydrogel sponge. *Bio-Med Mater Eng* **13**: 309–316.

Athanasiou KA, Niederauer GG, Agrawal CM. 1996; Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid polyglycolic acid copolymers. *Biomaterials* **17**: 93–102.

Barry F, Boynton RE, Liu B, *et al*. 2001; Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. *Exp Cell Res* **268**: 189–200.

Barry JJA, Gidda HS, Scotchford CA, *et al*. 2004; Porous methacrylate scaffolds: supercritical fluid fabrication and *in vitro* chondrocyte responses. *Biomaterials* **25**: 3559–3568.

Benya PD, Shaffer JD. 1982; Dedifferentiated chondrocytes re-express the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**: 215–224.

Brittberg M, Lindahl A, Nilsson A, *et al*. 1994; Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* **331**: 889–895.

Caplan AI. 1991; Mesenchymal stem cells. *J Orthopaed Res* **9**: 641–650.

Deschaseaux F, Gindraux F, Saadi R, *et al*. 2003; Direct selection of human bone marrow mesenchymal stem cells using an anti-CD49a

antibody reveals their CD45 (med, low) phenotype. *Br J Haematol* **122**: 506–517.
Dominici M, Le Blanc K, Mueller I, *et al*. 2006; Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**: 315–317.
Estes BT, Wu AW, Guilak F. 2006; Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. *Arthrit Rheumat* **54**: 1222–1232.
Fehrer C, Lepperdinger G. 2005; Mesenchymal stem cell aging. *Exp Gerontol* **40**: 926–930.
Francioli SE, Martin I, Sie CP, *et al*. 2007; Growth factors for clinical-scale expansion of human articular chondrocytes: relevance for automated bioreactor systems. *Tissue Eng* **13**: 1227–1234.
Freed LE, Hollander AP, Martin I, *et al*. 1998; Chondrogenesis in a cell–polymer bioreactor system. *Exp Cell Res* **240**: 58–65.
Freed LE, Marquis JC, Nohria A, *et al*. 1993; Neocartilage formation *in vitro* and *in vivo* using cells cultured on synthetic biodegradable polymers. *J Biomed Mater Res* **27**: 11–23.
Guo CA, Liu XG, Huo JZ, *et al*. 2007; Novel gene-modified tissue engineering of cartilage using stable transforming growth factor β1-transfected mesenchymal stem cells grown on chitosan scaffolds. *J Biosci Bioeng* **103**: 547–556.
Hennig T, Lorenz H, Thiel A, *et al*. 2007; Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFβ receptor and BMP profile and is overcome by BMP-6. *J Cell Physiol* **211**: 682–691.
Hofmann S, Knecht S, Langer R, *et al*. 2006; Cartilage-like tissue engineering using silk scaffolds and mesenchymal stem cells. *Tissue Eng* **12**: 2729–2738.
Homicz MR, Schumacher BL, Sah RL, *et al*. 2002; Effects of serial expansion of septal chondrocytes on tissue-engineered neocartilage composition. *Otolaryngol Head Neck Surg* **127**: 398–408.
Hwang NS, Varghese S, Zhang Z, *et al*. 2006; Chondrogenic differentiation of human embryonic stem cell-derived cells in arginine–glycine–aspartate-modified hydrogels. *Tissue Eng* **12**: 2695–2706.
Im GI. 2005; Chondrogenesis from mesenchymal stem cells derived from adipose tissue on the fibrin scaffold. *Curr Appl Physics* **5**: 438–443.
Im GI, Shin YW, Lee KB. 2005; Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? *Osteoarthrit Cartilage* **13**: 845–853.
Indrawattana N, Chen GP, Tadokoro M, *et al*. 2004; Growth factor combination for chondrogenic induction from human mesenchymal stem cell. *Biochem Biophys Res Commun* **320**: 914–919.
Jin XB, Sun YS, Zhang K, *et al*. 2007; Neocartilage formation from predifferentiated human adipose derived stem cells *in vivo*. *Acta Pharmacol Sin* **28**: 663–671.
Jukes JM, Moroni L, van Blitterswij KCA, *et al*. 2008; Critical steps toward a tissue-engineered cartilage implant using embryonic stem cells. *Tissue Eng A.* **14**: 135–147.
Kameda T, Koike C, Saitoh K, *et al*. 2000; Analysis of cartilage maturation using micromass cultures of primary chondrocytes. *Dev Growth Different* **42**: 229–236.
Karageorgiou V, Kaplan D. 2005; Porosity of 3D biornaterial scaffolds and osteogenesis. *Biomaterials* **26**: 5474–5491.
Karp JM, Ferreira LS, Khademhosseini A, *et al*. 2006; Cultivation of human embryonic stem cells without the embryoid body step enhances osteogenesis *in vitro*. *Stem Cells* **24**: 835–843.
Kim UJ, Park J, Kim HJ, *et al*. 2005; Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin. *Biomaterials* **26**: 2775–2785.
Kisiday JD, Kopesky PW, Evans CH, *et al*. 2008; Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. *J Orthopaed Res* **26**: 322–331.
Koay EJ, Hoben GMB, Athanasiou KA. 2007; Tissue engineering with chondrogenically differentiated human embryonic stem cells. *Stem Cells* **25**: 2183–2190.
Lahiji A, Sohrabi A, Hungerford DS, *et al*. 2000; Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. *J Biomed Mater Res* **51**: 586–595.
Langer R, Vacanti JP. 1993; Tissue Engineering. *Science* **260**: 920–926.
Lohmander LS. 2003; Tissue engineering of cartilage: do we need it, can we do it, is it good and can we prove it? *Novartis Found Sympos* **249**: 2–10.
Malafaya PB, Pedro AJ, Peterbauer A, *et al*. 2006; Chitosan particles agglomerated scaffolds for cartilage and osteochondral tissue engineering approaches with adipose tissue derived stem cells. *J Mater Sci Mater Med* **17**: 675–675.
Mallein-Gerin F, Ruggiero F, Garrone R. 1990; Proteoglycan core protein and type II collagen gene expressions are not correlated with cell shape changes during low density chondrocyte cultures. *Differentiation* **43**: 204–211.
Mastrogiacomo M, Derubeis AR, Cancedda R. 2005; Bone and cartilage formation by skeletal muscle derived cells. *J Cell Physiol* **204**: 594–603.
Park J, Gelse K, Frank S, *et al*. 2006; Transgene-activated mesenchymal cells for articular cartilage repair: a comparison of primary bone marrow-, perichondrium/periosteum- and fat-derived cells. *J Gene Med* **8**: 112–125.
Pittenger MF, Mackay AM, Beck SC, *et al*. 1999; Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–147.
Sechriest VF, Miao YJ, Niyibizi C, *et al*. 2000; GAG-augmented polysaccharide hydrogel: a novel biocompatible and biodegradable material to support chondrogenesis. *J Biomed Mater Res* **49**: 534–541.
Sekiya I, Colter DC, Prockop DJ. 2001; BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. *Biochem Biophys Res Commun* **284**: 411–418.
Sittinger M, Reitzel D, Dauner M, *et al*. 1996; Resorbable polyesters in cartilage engineering: affinity and biocompatibility of polymer fiber structures to chondrocytes. *J Biomed Mater Res* **33**: 57–63.
Sottile V, Halleux C, Bassilana F, *et al*. 2002; Stem cell characteristics of human trabecular bone-derived cells. *Bone* **30**: 699–704.
Suh H. 1998; Recent advances in biomaterials. *Yonsei Med J* **39**: 87–96.
Suh JKF, Matthew HWT. 2000; Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. *Biomaterials* **21**: 2589–2598.
Sze SK, de Kleijn DPV, Lai RC, *et al*. 2007; Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. *Mol Cell Proteom* **6**: 1680–1689.
Thomson JA. 1998; Embryonic stem cell lines derived from human blastocysts. *Science* **282**: 1827–1827.
Tığlı RS, Karakecili A, Gumusderelioglu M. 2007; *In vitro* characterization of chitosan scaffolds: influence of composition and deacetylation degree. *J Mater Sci Mater Med* **18**: 1665–1674.
Tuan RS, Boland G, Tuli R. 2003; Adult mesenchymal stem cells and cell-based tissue engineering. *Arthrit Res Therapy* **5**: 32–45.
Velema J, Kaplan D. 2006; Biopolymer-based biomaterials as scaffolds for tissue engineering. *Tissue Eng I Scaffold Syst Tissue Eng* **102**: 187–238.
von der Mark K, Gauss V, von der Mark H, *et al*. 1977; Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* **267**: 531–532.
Wakitani S, Goto T, Pineda SJ, *et al*. 1994; Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg Am* **76A**: 579–592.
Wakitani S, Imoto K, Yamamoto T, *et al*. 2002; Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. *Osteoarthrit Cartilage* **10**: 199–206.
Wang YZ, Blasioli DJ, Kim HJ, *et al*. 2006; Cartilage tissue engineering with silk scaffolds and human articular chondrocytes. *Biomaterials* **27**: 4434–4442.
Wang YZ, Kim UJ, Blasioli DJ, *et al*. 2005; *In vitro* cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. *Biomaterials* **26**: 7082–7094.
Williams CG, Kim TK, Taboas A, *et al*. 2003; *In vitro* chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. *Tissue Eng A* **9**: 679–688.
Winter A, Breit S, Parsch D, *et al*. 2003; Cartilage-like gene expression in differentiated human stem cell spheroids – a comparison of bone marrow-derived and adipose tissue-derived stromal cells. *Arthrit Rheumat* **48**: 418–429.
Wobus AM, Holzhausen H, Jakel P, *et al*. 1984; Characterization of a pluripotent stem-cell line derived from a mouse embryo. *Exp Cell Res* **152**: 212–219.

Wu M, Yang L, Liu S, *et al*. 2006; Differentiation potential of human embryonic mesenchymal stem cells for skin-related tissue. *Br J Dermatol* **155**: 282–291.

Yoshimura H, Muneta T, Nimura A, *et al*. 2007; Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res* **327**: 449–462.

Zhao J, Yuan XY, Cui YL, *et al*. 2004; Preparation and characterization of poly(L-lactide)/poly(ε -caprolactone); fibrous scaffolds for cartilage tissue engineering. *J Appl Polymer Sci* **91**: 1676–1684.

Zuk PA, Zhu M, Mizuno H, *et al*. 2001; Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**: 211–228.