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Article

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# Directing Chondrogenesis of Stem Cells with Specific Blends of Cellulose and Silk

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## ABSTRACT

Biomaterials that can stimulate stem cell differentiation without growth factor supplementation provide potent and cost-effective scaffolds for regenerative medicine. We hypothesize that a scaffold prepared from cellulose and silk blends can direct stem cell chondrogenic fate. We systematically prepared cellulose blends with silk at different compositions using an environmentally benign processing method based on ionic liquids as a common solvent. We tested the effect of blend compositions on the physical properties of the materials as well as on their ability to support mesenchymal stem cell (MSC) growth and chondrogenic differentiation. The stiffness and tensile strength of cellulose was significantly reduced by blending with silk. The characterized materials were tested using MSCs derived from four different patients. Growing MSCs on a specific blend combination of cellulose and silk in a 75:25 ratio significantly upregulated the chondrogenic marker genes SOX9, aggrecan and type II collagen in the absence of specific growth factors. This chondrogenic effect was neither found with neat cellulose nor the cellulose/silk 50:50 blend composition. No adipogenic or osteogenic differentiation is detected on the blends suggesting that the cellulose/silk 75:25 blend induces specific stem cell differentiation into the chondrogenic lineage without addition of the soluble growth factor TGF- $\beta$ . The cellulose/silk blend we identified can be used both for *in vitro* tissue engineering and as an implantable device for stimulating endogenous stem cells to initiate cartilage repair.

KEYWORDS: cellulose, silk, ionic liquids, chondrogenesis, stem cell, cartilage tissue engineering

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3 **1. Introduction**  
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5 Mesenchymal stem cells (MSCs) have been widely used in cartilage tissue engineering  
6 studies<sup>1, 2</sup>. We have used MSCs to create a tissue-engineered trachea that was grown in a  
7 bioreactor<sup>3</sup> and then successfully implanted this in a patient with bronchomalacia<sup>4</sup>. This  
8 method required the use of at least three growth factors, including TGF- $\beta$ <sup>5</sup>, and extensive *ex*  
9 *vivo* cell culture. Most of the current materials known to support MSC chondrogenesis depend  
10 on the addition of one or more growth factors from the TGF- $\beta$  superfamily to stimulate MSC  
11 differentiation and matrix deposition. Materials with the property of directing specific MSC  
12 differentiation in the absence of growth factors would simplify *in vitro* tissue engineering  
13 procedures and could be implanted into patients without added cells in order to initiate tissue  
14 regeneration through activation of endogenous MSCs.  
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30 Many studies have established that complex interactions between soluble and extracellular  
31 matrix molecules regulate intracellular signaling and differentiation. Although direct activation  
32 of signal transduction by matrix molecules through integrin receptors has been well studied, the  
33 physical properties of the matrix, such as its elasticity or stiffness, are also important<sup>6, 7</sup>. In  
34 order to progress with controlling stem cell fate without dependence on soluble factors, custom-  
35 engineered artificial or natural materials with controlled surface and biomechanical properties  
36 can be developed. One method for achieving this objective involves blending biocompatible  
37 materials with known properties to support stem cell fate. Recently, blends of artificial and  
38 natural polymers have been used to support the chondrogenic differentiation of MSCs<sup>8</sup>. In all  
39 cases, the use of chondrogenic growth factors remained essential for driving MSC differentiation  
40 and matrix deposition.  
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Cellulose, which is a linear homopolymer of glucose ( $C_6H_{10}O_5$ )<sub>n</sub> with n ranging from 500 to 5000, is the most abundant polymer in nature. It is degradable by enzymes and its solubility in water depends on its chain length n<sup>9</sup>. Cellulose is easily fabricated and thus available in a wide range of forms and shapes, e.g. as membrane sponges, microspheres and non-woven, woven or knitted textiles. The biocompatibility and robust mechanical properties of cellulose and its derivatives is well established<sup>10, 11</sup>. Unlike synthetically-produced biomaterials, such as polylactic acid and polyglycolic acid which are known to induce inflammation secondary to the production of acidic residues during degradation<sup>12-15</sup>, cellulose degrades to yield glucose. As a consequence, cellulose has been used to support embryonic stem cell growth<sup>16</sup>, neural differentiation of mesenchymal stem cells<sup>17</sup> and retinal stem-progenitor cell survival and proliferation<sup>18</sup>.

To date, however, the potential of cellulose for inducing stem cell chondrogenesis has not been investigated. Cellulose, which comprises three hydroxyl groups per repeating unit, is theoretically a good choice as an initiator of chondrogenesis. The presence of hydroxyl groups on the surface of a biomaterial has been shown to encourage chondrogenic differentiation of stem cells<sup>19</sup>. On the other hand, silk is widely used as a biomaterial for tissue engineering applications due to its toughness<sup>20-24</sup>. The fibroin protein of silk is composed of a 59-mer amino acid repeat sequence organized as pleated sheets<sup>25</sup>. Silk and its derivatives can support MSC chondrogenesis in the presence of chondrogenic growth factors<sup>26-28</sup>. By blending cellulose, which is a stiff polymer, in different proportions with silk, it should be possible to change the stiffness of the blends and the proportion of hydroxyl and amide functional groups present in a substrate. In support of this hypothesis, a few studies have demonstrated a reduction in the mechanical properties of cellulose upon blending with silk. Freddi et al. reported a greater than

two-fold reduction in the tensile strength of cellulose after blending with silk at 2:3 ratio <sup>29</sup>. Kuzmina et al. prepared cellulose and silk blend films using 1-butyl-3-methylimidazolium chloride, and found the tensile strength of neat cellulose to reduce from 50.1 MPa to 31.2 MPa in the presence of silk at 90% <sup>30</sup>. Finally, Hirano et al. used a viscose-based process to prepare cellulose and silk blend fibres with silk content ranging from 0 to 53wt%. They observed a decrease in the tenacity (strength) of the fibres from 1.27 g/denier for neat cellulose to 0.15 g/denier for 53wt% of silk <sup>31</sup>.

Despite having these advantages, widescale use of cellulose in stem cell-based tissue engineering remains relatively restricted due in part to the lack of a simple, environmentally benign processing method for manufacturing it into useable biomaterials. Examples of using cellulose with stem cells include preparing bacterial cellulose sponges that support stem cell proliferation <sup>32</sup>, osteogenic differentiation of stem cells on hydroxyapatite-coated bacterial cellulose <sup>33</sup> and maintenance of myoblasts on cellulose nano-whiskers <sup>34, 35</sup>. Cellulose degrades before it melts, and therefore it cannot be melt processed. In addition, solvents traditionally used for solution casting/spinning cellulose, such as carbon disulphide and sulphuric acid, are highly aggressive and hazardous. Ionic liquids have emerged as a new class of environmentally benign solvents which can effectively dissolve natural polymers such as cellulose, silk and chitin <sup>36-39</sup>, to prepare films <sup>38, 40, 41</sup>, fibres/nanofibres <sup>36, 42-45</sup>, gels <sup>41, 46-48</sup> and foams <sup>47, 49</sup>.

In this report, we demonstrate for the first time that a specific blend of cellulose and silk, prepared using ionic liquids, provides self-supported membranes with the ability to initiate the chondrogenic differentiation of human MSCs in the absence of chondrogenic growth factors.

## 2. Experimental Section

*2.1 Preparation of the natural polymer and blends.* Cellulose from wood pulp (degree of polymerization, 900) was purchased from Rayonier Inc. (Jacksonville, FL, USA). *Bombyx mori* silk (obtained from Aurora Silk, Portland, OR, USA) was boiled in 0.02M Na<sub>2</sub>CO<sub>3</sub> solution and washed before dissolution and preparation of membranes. The ionic liquid, 1-ethyl-3-methylimidazolium acetate (EMI Ac) obtained from Sigma Aldrich, was used as a solvent for cellulose and silk. All the tissue culture chemicals were purchased from Sigma unless otherwise stated. The 2D membranes of cellulose, and its blends with silk were produced using the following procedure: 1.5% (w/w) of cellulose was dissolved in 5 g of EMI Ac in a glass vial with continuous stirring and heating at 80 °C. The heating was carried out for 2 h to ensure complete dissolution. Subsequently, silk was added to above solution. The amount of cellulose and silk were varied to achieve desired cellulose to silk weight fractions in the final membrane. The cellulose solution was poured into a glass petri dish and allowed to cool for 3 h. The cooled solution was coagulated by pouring ethanol into the petri dish. Ethanol was added to the membranes to selectively dissolve the EMI Ac and coagulate the cellulose membrane. The coagulated membrane was soaked in distilled water for two days to remove any trace of EMI Ac. The membranes were dried at room temperature to remove the water. To prepare silk and cellulose blends, an appropriate amount of silk was dissolved using EMI Ac as a common solvent. For the effective coagulation of silk, a mixture of ethanol and acetic acid (90:10) was used<sup>50</sup>. The rest of the membranes were prepared using the same process described above. In total, 3 materials were prepared: cellulose alone (100%), cellulose/silk (75:25), cellulose/silk (50:50). Preparation of blends with more than 50% silk and pure regenerated silk produced brittle membranes that were difficult to handle without breaking. A similar reduction in the



mechanical properties of cellulose/silk blends<sup>51, 52</sup> and pure regenerated silk<sup>53</sup> was also reported previously. The films' thickness measured approximately 7-10  $\mu\text{m}$ .

*2.2 Chemical and mechanical characterization.* The chemical composition of the pure cellulose, and its blends with silk, was assessed using Fourier transform infrared spectroscopy (FTIR) analysis. The analysis was carried out in transmission mode using a Spectrum 100 FTIR spectrometer (PerkinElmer, Waltham, MA, USA). Scanning electron microscopy (SEM) imaging was performed using a field emission gun scanning electron microscope (JEOL Ltd., Tokyo, Japan) with an accelerating voltage of 15.0 kV and working distances between 15 mm and 6 mm. Thin 2D membranes of cellulose and its blends with silk were fixed to an aluminum stub with carbon pad. In order to avoid surface charging gold was sputtered onto the samples with an EMITECH sputter coater. Raman Spectroscopy was carried out using a Renishaw Ramanscope 1000 system with at least 10 spectra being collected at different positions along the length of each sample. Here HeNe laser was used (wavelength = 633 nm) with a laser spot size of 10 microns. AFM images were taken using either a Bruker (formerly Veeco) Dimension 3100 or Bruker Multimode IIIa. Images were taken in tapping mode in air, using AppNano ATC-25 silicon cantilevers with a nominal tip radius of 10 nm and resonant frequency of 307 kHz. Drive amplitudes were often high to combat surface adhesion, but set points were kept at 80% of free amplitude. Two and ten micron height and phase images, 512 x 512 pixels in resolution, were collected. Mechanical testing of the membranes was carried out using a Textechno Favimat (Mönchengladbach, Germany) that is ideally suited for measuring the tensile strength, stiffness and linear density of very thin samples such as fibres and films. Membranes were cut into 20 mm x 3 mm pieces and gripped between two jaws of the Favimat testing machine. The sample was pulled apart until failure at an elongation rate of 2 mm min<sup>-1</sup> for all of the tests. Readings for

linear density via mechanical resonance measurements enabled the data to be plotted as specific strength and specific stiffness (expressed in  $\text{cN Tex}^{-1}$ , which is numerically equivalent to  $\text{GPa SG}^{-1}$ , where  $\text{Tex} = \text{g km}^{-1}$  and SG is specific gravity). The stress ( $\text{cN/tex}$ ) and strain (%) displacement of sample) data were logged using the data acquisition and analysis software provided by Favimat. The stress was calculated by dividing applied load value by linear density of the sample ( $\text{tex}$ ), while the ultimate tensile strength ( $\text{cN/tex}$ ) was calculated as maximum load ( $\text{cN}$ ) before sample failure divided by the linear density of the film ( $\text{tex}$ ). The slope of the stress-strain curve generated was used to calculate the stiffness ( $\text{cN/tex}$ ) of the sample.

**2.3 Cell culture.** Bone marrow plugs were collected from the femoral heads of patients ( $n=4$ ) undergoing complete replacement hip arthroplasty. All patients provided informed consent and the study was carried out according to local ethical guidelines. Cells were suspended in stem cell expansion medium consisting of low glucose Dulbecco's Modified Eagles Medium supplemented with 10 % (v/v) Fetal Bovine Serum (FBS, Thermo Scientific Hyclone, Loughborough, UK), 1% (v/v) Glutamax (Sigma, Poole, UK) and 10 % (v/v) Penicillin G (10,000 units/ml)/Streptomycin (10,000 mg/ml) antibiotic mixture (P/S; Sigma) P/S. The serum batch was selected to promote the growth of MSCs<sup>54</sup>. The medium was also supplemented with 2 ng/ml fibroblast growth factor 2 (FGF-2, PeproTech, London, UK) to enhance MSCs proliferation<sup>55</sup>. The cell suspension was separated from any bone in the sample by repeated washing with media. The cells were centrifuged at 500 g for 5 min and the supernatant/fat removed. The resulting cell pellet was resuspended in medium, and then plated at a seeding density of between  $1.5$  and  $2.0 \times 10^5$  nucleated cells per  $\text{cm}^2$ . These flasks were incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  and 95% air. Four days were allowed before the first medium change and then the medium was changed every other day until adherent cells reached

90% confluence and were ready for passaging. All experiments were done with passage three cells.

*2.4 Preparation of 2D membranes for cell seeding.* The dry cellulose and cellulose/silk blends were cut as circular discs with an 8 mm diameter biopsy punch and placed in a 24-well tissue culture plate. They were disinfected with 70% (v/v) ethanol for 30 minutes and washed a few times with sterile phosphate buffered saline (PBS). Standard tissue culture plastic (Corning) was used as a control for cell adherence. The polystyrene surface has been modified using corona discharge to make the surface hydrophilic and negatively charged when medium is added<sup>56</sup>. The expansion medium used for growing MSCs on this surface is accepted as a criterion for maintaining the multipotent state of MSCs<sup>57</sup>. The membranes or plastic were coated with fibronectin (100 µg/ml; Sigma) for 5 h at 37 °C, washed with PBS and transferred to an ultralow attachment plate to dry overnight.

*2.5 Cell seeding and culture.* The cells were loaded on the fibronectin-coated materials at a density of  $28 \times 10^3$  cells per  $\text{cm}^2$ . The seeded cells were cultured in expansion medium as described above with FGF-2 at 10 ng/ml. Cells seeded on plastic as positive controls were maintained in the same medium. The medium was changed twice a week. The cells were incubated for 14 days before downstream analysis.

*2.6 Cell adhesion and viability assay.* Live monitoring of cell adhesion and viability was conducted using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Paisley, UK) as per manufacturer instructions. Cell-loaded materials were incubated for 72 h at 37 °C in a humidified atmosphere of 5%  $\text{CO}_2$  and 95% air. The constructs were washed with PBS and incubated with the kit reagent that stains live cells with green fluorescent dye (calcein AM, emission 488nm) and dead cells with red fluorescent dye (ethidium homodimer-1, emission

568nm). Negative controls consisting of cells killed with methanol and positive controls consisting of cells grown on a tissue culture plastic plate were run with each set of experiments. The plates were viewed under a widefield fluorescence microscope system (Leica DMIRB inverted microscope, Houston, TX, USA).

*2.7 Quantitative real-time polymerase chain reaction.* Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Netherlands). RNA concentration and purity were determined spectrometrically at 260 and 280 nm. Complementary DNA (cDNA) was synthesized using Takara Primescript 1st Strand cDNA Synthesis kit (Shiga, Japan) according to the manufacturer's protocol. Quantitative real time polymerase chain reaction (qRT-PCR) was performed as described previously<sup>5, 54</sup>. A 25  $\mu$ l reaction consisted of 12.5  $\mu$ l of the SYBR Premix Ex Taq (Perfect Real Time; Takara), 5  $\mu$ l of the cDNA reaction mixture, and 300 nM primers using the Rotorgene 6000 Cyclor (Qiagen, Crawley, UK). The amplification programme consisted of initial denaturation at 95 °C (2 min) followed by 40 cycles of denaturation at 95 °C (15 s) and annealing/extension at 58 °C (30 s). After amplification, melt analysis was performed by heating the reaction mixture from 60 °C to 95 °C at a rate of 0.2 °C/s. The cycle threshold (Ct) value for each gene of interest was measured for each sample. The Ct value for  $\beta$ -Actin was used as an endogenous reference for normalization. Real time RT-PCR assays were done in duplicate or triplicate and repeated two to four times. Primers for cartilage specific genes were designed and optimized as previously described<sup>5, 54</sup>. The sequences of the primers used were

SOX9 (F) CTTTGGTTTGTGTTTCGTGTTTTG, SOX9 (R) AGAGAAAGAAAAAGGGAA  
AGGTAAGTTT, Aggrecan (F) AGGGCGAGTGGAATGATGTT, Aggrecan (R) GG  
TGGCTGTGCCCTTTTAC, Collagen II  $\alpha$ 1 (A+B) (F) CAACACTGCCAACGTCCAGAT,  
Collagen II  $\alpha$ 1 (A+B) (R) CTGCTTCGTCCAGATAGGCAAT, Collagen I  $\alpha$ 2(I) (F) TCT GGA

TGG ATT GAA GGG ACA, Collagen I  $\alpha 2(I)$  (R) CCA ACA CGT CCT CTC TCA CC,  $\beta$ -Actin (F) GACAGGATGCAGAAGGAGATTACT,  $\beta$ -Actin (R) TGATCCACATCTGCTGGAAGGT.

*2.8 Assessment of MSC multipotential.* Chondrogenic differentiation on blends or plastic was stimulated by adding differentiation medium consisting of DMEM containing 4.5 g/l glucose supplemented with 10 ng/ml of transforming growth factor-3 (TGF- $\beta 3$ ; R&D Systems), 1 mM sodium pyruvate (Sigma), 50  $\mu$ g/ml ascorbic acid-2-phosphate (Sigma),  $1 \times 10^{-7}$  M dexamethasone (Sigma), 1% ITS (Invitrogen), and 1% (v/v) Penicillin (100 U/ml) /Streptomycin (100  $\mu$ g/ml) (Invitrogen). Medium was changed every 2 to 3 days. Negative controls were incubated in differentiation medium without TGF- $\beta 3$ . In all cultures, the medium was replaced every 3 to 4 days for a period of 21 days. The cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde at room temperature. The cells were permeabilized with 1% (w/v) BSA in PBS containing 10% (v/v) normal donkey serum and 0.3% (v/v) Triton X-100 at room temperature. After blocking with 1% BSA/PBS, the films were incubated with goat anti-human aggrecan antibody (R&D systems) or mouse anti-human type II collagen antibody (Santa Cruz) overnight at 2-8  $^{\circ}$ C. The films were incubated with fluorescent secondary antibody (NorthernLights 557 Fluorochrome-conjugated donkey anti-goat secondary antibody; R&D Systems) for 60 minutes at room temperature. The membranes were washed with 1% BSA/PBS and images taken using a Leica widefield fluorescence microscope. Negative controls included samples incubated with or without normal donkey serum at the appropriate corresponding concentrations of the primary antibodies. For osteogenic positive controls MSCs were grown to 50-70% confluency on blend membranes and plastic and incubated in osteogenic medium containing 100 nM dexamethasone, 0.2 mM ascorbic acid and 10 mM  $\beta$ -glycerolphosphate

(Sigma). Negative controls were cultured without  $\beta$ -glycerolphosphate stimulation. In all cultures, the medium was replaced every 3 to 4 days for a period of 21 days. The cells were then washed with PBS, fixed in a solution of ice-cold 70% ethanol for 1 h, and stained for 10 min with 1 ml of 40 mM Alizarin red (pH 4.1; Sigma). For adipogenic differentiation, 50-70% confluent BMSCs were incubated in complete medium supplemented, in positive control cultures, with 0.5  $\mu$ M hydrocortisone, 0.5 mM isobutyl-methylxanthine and 60  $\mu$ M indomethacin (all from Sigma). Negative controls were cultured in complete medium without the supplements. In all cultures, the medium was replaced every 3 to 4 days for a period of 21 days. Cells were washed with PBS, fixed in 10% formalin for 10 min, and stained for 15 min with fresh Oil Red-O solution (Sigma).

*2.9 Statistical analysis.* All experiments were done with individual patient samples in duplicate or triplicate. The sample sizes  $n$  reflects the number of individual patient samples used for each experiment. Comparison of differences between individual groups was by Student's  $t$ -test. Multiple group comparisons were made using either analysis of variance using (ANOVA) or the non-parametric Kruskal–Wallis test. Where significant variance was demonstrated, differences between individual groups were determined using the Bonferroni correction or Dunn's test *post hoc*, as appropriate. In all cases,  $p < 0.05$  was taken as significant.

### 3. Results

**3.1 Scanning electron microscopy.** Blends of cellulose and silk at various ratios were prepared as described above. The blends were fabricated as thin membranes that were cut into 8 mm discs (Figure 1A). Scanning electron microscopy (SEM) analysis was carried out to

investigate the surface structure and morphology of membranes prepared from pure cellulose and its blends with silk (Figure 1 B-G). In each case, the surface of the membranes appeared to be rough with no observable difference between blends. Importantly, cross-sections of the films showed no indication of phase separation of the blends proving homogenous dispersion of cellulose and silk within the membranes.

**3.2 FTIR Analysis.** The chemical compositions of the cellulose and cellulose/silk hybrid membranes were studied using FTIR analysis. Figure 2 (A) shows a comparison of the FTIR data for regenerated pure cellulose, cellulose/silk 75:25 blend, cellulose/silk 50:50 blend and regenerated pure silk. The pure cellulose does not have an amide group in its molecular structure, hence it does not show amide peaks, but as silk is added amide peaks begin to appear. Thus the spectrum of the cellulose/silk 75:25 blend showed an amide I peak at  $1624\text{ cm}^{-1}$  and an amide II peak at  $1530\text{ cm}^{-1}$  which are signature peaks of the silk component<sup>58</sup>. The cellulose/silk 50:50 blend also showed amide I and amide II peaks at the slightly lower frequencies of  $1621\text{ cm}^{-1}$  and  $1515\text{ cm}^{-1}$ , respectively. These data demonstrate that the blending had no effect on the inherent chemical functionalities associated with cellulose and silk.

**3.3 Raman Spectroscopy and AFM.** A potential limitation when using cellulose as a substrate for MSCs is its inert capacity in not adhering this type of cell. In order to overcome this limitation, the membranes were precoated with fibronectin, an extracellular matrix protein known to enhance MSC adhesion to biomaterials<sup>5</sup>. Raman spectroscopy was carried out in order to probe the effect of fibronectin coating on the availability of the functional groups present in the membrane. Figure 3 shows the Raman spectra of cellulose (Figure 3, A-B), cellulose/silk 75:25 (Figure 3, C-D) and cellulose/silk 50:50 (Figure 3, E-F) blends with fibronectin coating. In each case, the samples clearly show a distinct peak at  $2889\text{ cm}^{-1}$  which

correspondences to CH and CH<sub>2</sub> bond stretching peak in cellulose<sup>59</sup>. Similarly, the cellulose glycosidic ring breathing mode peak at 1095cm<sup>-1</sup><sup>60</sup> is present. The Raman spectra can probe the depth up to a few hundred nanometers<sup>61</sup>. Since the cells will interact with the membranes in the wet state, it was hypothesized that fibronectin conformation may change. Figure 3 demonstrates there was no detectable difference between the cellulose peaks in the dry and wet states. Taken together, the results clearly demonstrate that motioned functional groups can be detected by Raman spectroscopy techniques even after coating with fibronectin in dry and wet states, indicating that fibronectin does not mask the functional groups present in the membranes.

We considered it important to analyze the topography and surface roughness of the membranes at the highest resolution possible. To this end, we employed AFM imaging for all membranes (Figure 4). Typical images of cellulose films showed granular surface morphology (Figure 4A). However, doping of silk had a significant effect on the surface topography of the films. As silk concentration was increased films exhibited smooth surface morphology (Figure 4, B and C). The data suggests that cellulose/silk blending impacts the surface topography at the nanolevel.

**3.4 Mechanical properties.** We hypothesize that cellulose and silk blends can direct stem cell fate in part due their elastic properties<sup>7</sup>. The mechanical properties of all the regenerated polymer membranes were tested using a Favimat testing machine, as described above. Figure 5 (A) shows the stiffness (elasticity) of pure cellulose and its blends with silk. Statistically significant decreases in the stiffness of cellulose/silk blends were observed as compared to the pure cellulose polymer. Figure 5 (B) shows the comparison of the tensile strength of pure cellulose and the 75:25 and 50:50 cellulose/silk blends. A significant reduction in the tensile strength was observed in the cellulose/silk blends as compared to pure cellulose polymer.



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**3.5 MSC viability and morphology.** MSCs seeded on each of the blends were tested for viability and growth using the LIVE/DEAD<sup>®</sup> viability assay. Cell morphology was assessed by microscopic analysis. It is hypothesized since pure silk can encourage MSC adhesion<sup>28</sup>, cellulose/silk blends may attain this capacity. Initial experiments were conducted to assess the adhesion and viability of MSCs on all blends without fibronectin coating. There was a very weak improvement in the adhesion of MSCs with an increase in the silk concentration of the blends (data not shown); however this weak improvement was not sufficient to eliminate the need for fibronectin. From this point onwards, fibronectin coating was used to facilitate MSC adhesion onto the membranes. A high number of live cells (green) attached to the membranes after 3 days and they continued to grow for 14 days (Figure 6). A negligible number of dead cells (red) were detected irrespective of the polymer, blend or time point. Positive controls using cells seeded on plastic (Figure 6 G-H) or negative controls using cells killed by methanol (Figure 6 I) are shown for comparison. The cells maintained their typical fibroblastic morphology on pure cellulose (Figure 6 A-B) similar to plastic (Figure 6 G-H) suggesting maintenance of the undifferentiated phenotype on cellulose. On the other hand, the cells assumed a more diffuse and dense appearance on the blends (Figure 6 C-F and I-L). The observed change of phenotype for MSCs grown on the blends suggested these have undergone auto-differentiation.

**3.6 Chondrogenic induction.** In order to assess the multipotential of MSCs seeded on the blends, qPCR analysis for chondrogenic, adipogenic and osteogenic marker genes was conducted. Figure 7 shows qPCR analysis for the chondrogenic markers, SOX9, aggrecan, and type II collagen. Type I collagen, which is not normally synthesized by chondrocytes, was included to assess the degree of chondrocyte dedifferentiation. There was a small upregulation of chondrogenic marker genes aggrecan, type II collagen and SOX9 for MSCs grown on pure

cellulose compared to cells grown on plastic. This upregulation increased to a significant level when cells were grown on the cellulose/silk 75:25 blend membrane (Figure 7). Type I collagen was not significantly upregulated under any condition. The ratio of collagen II to collagen I is a measure of chondrogenic quality with a higher level indicating more chondrogenesis and less dedifferentiation. This ratio was significantly higher for MSCs grown on the cellulose/silk 75:25 blend than on any other material. Analysis of gene expression for the osteogenic marker osteocalcin, and the adipogenic marker, adipose most abundant gene transcript-1 (AMP-1), showed no detectable signal by RT-PCR analysis (data not shown) suggesting selective commitment of MSCs to the chondrogenic lineage on the cellulose/silk 75:25 blend.

**3.7 Cartilage formation.** The deposition of the extracellular matrix proteins, type II collagen and aggrecan is a hallmark of committed hyaline chondrocytes. In order to confirm that the observed upregulation of chondrogenic genes is not transient, MSCs were grown on the cellulose/silk 75:25 blend membrane for 21 days then stained for type II collagen and aggrecan. Figure 8 shows strong staining for aggrecan and type II collagen in the absence of the chondrogenic stimulation of TGF- $\beta$ . There was no detectable staining for MSCs grown on plastic. When MSCs on plastic were incubated with TGF- $\beta$ , strong staining was observed for aggrecan and type II collagen (Figure 8A). The results confirm that the cellulose/silk 75:25 blend is not only inductive of chondrogenesis as shown by qPCR but also a driver of chondrogenic MSC maturation and cartilage matrix deposition. The observed specific chondrogenic stimulation of MSCs by the cellulose/silk 75:25 blend was confirmed by assessing the blend's capacity for osteogenic and adipogenic induction. Figure 8B shows an absence of osteogenesis and adipogenesis, characterized by calcium deposition and the presence of fatty acid vacuoles, respectively. MSCs grown on plastic in the presence of soluble osteogenic and

adipogenic supplements showed strong staining for calcium and fatty acids, respectively. Taken together, the data confirms the specific capacity of the cellulose/silk 75:25 blend to direct MSCs into chondrogenic differentiation and not adipogenic or osteogenic lineages.

#### 4. Discussion

We have shown that a specific blend of cellulose and silk processed using an environmentally benign common solvent can stimulate human mesenchymal stem cell chondrogenic differentiation. In this way the stiffness and proportion of hydroxyl and amide groups in the membranes was systematically varied to provide a material composition that can be used as a growth-factor independent method for controlling MSC differentiation.

Smart biomaterials that can induce stem cell differentiation without the need for external stimuli such as growth factors could result in major advances in the field of regenerative medicine. Such smart materials can cut the cost of clinical delivery by minimizing the need for long-term MSC cultures in expensive growth factors. They can also facilitate cell-free therapeutic modalities for stimulating endogenous stem cells, an approach favored by regulatory bodies and industry. Previous studies have shown that engineering the physical/chemical properties and architecture of biomaterials can be tuned to enhance differentiation of stem cells to a specific lineage. For example, Dalby et al. designed a range of nanoscale patterns on the surface of stem cell scaffolds and showed that a specific configuration promotes osteogenic differentiation even in the absence of an osteogenic supplement<sup>62</sup>. Similarly, Engler et al. showed that stem cell lineage specification can be controlled solely by manipulation of scaffold elasticity<sup>7</sup>. In addition, the presence of specific chemical functional groups on the scaffold surface can play a role in lineage specification of stem cells in the absence of external growth factors. Curran et al. showed that silane-treated glass surfaces functionalized with carboxyl (–

COOH) and hydroxyl (–OH) groups initiated chondrogenic marker mRNA expression in MSCs in the absence of chondrogenic growth factors, whereas amine (–NH<sub>2</sub>) functional groups encouraged osteogenic differentiation of stem cells in the absence of osteogenic supplements<sup>19</sup>. Our current work builds on these earlier studies by showing significantly enhanced chondrogenic differentiation of MSCs when using a specific combination of cellulose and silk blend membrane even without use of the standard chondrogenic growth factor, TGF-β. We hypothesize that the cellulose/silk 75:25 blend provides an optimum combination of membrane elasticity and appropriate combination of hydroxyl and amino functional groups to enhance the chondrogenic differentiation of stem cells. The AFM analysis revealed peculiar topography and surface roughness for the cellulose/silk 75:25 blend that is intermediate between the pure cellulose and cellulose/silk 50:50 blends. This suggests the interaction of MSCs with the cellulose/silk 75:25 blend is influenced not just by its chemical composition and mechanical properties, but also by the nano profile of the surface<sup>62</sup>. Understanding the molecular signalling events associated with that profile remains to be investigated.

Despite their abundance in nature, excellent mechanical properties and biocompatibility<sup>63-68</sup>, the use of cellulose in tissue engineering applications has been limited in part due to the lack of an environmentally benign processing method. Our approach for using ionic liquids both as common, environmentally benign solvents to dissolve pure cellulose and for blending with silk may help overcome any reluctance to use these natural materials as scaffolds for chondrogenic differentiation of stem cells. A potential drawback of the ionic liquid process is that it is coagulation based and diffusion of the liquid from coagulated biopolymers can take a long time. However this approach remains safer and easier to control than the traditionally used viscose and N-methylmorpholine-N-oxide process<sup>10</sup>.

Cellulose is a macromolecule of glucose monomers, which are naturally occurring biochemicals in the human body, hence its release from degrading cellulose *in vivo* is less likely to cause inflammation than common artificial materials such as polyglycolic acid and polylactic acid<sup>12-15</sup>. The contribution of glucose, as a cellulose degradation product, to the chondrogenic differentiation process of MSCs remains to be established. It is worth noting however that the standard media recipe for driving MSC chondrogenesis includes a high glucose concentration<sup>69</sup>.

Our results indicate that preferential stem cell differentiation on substrates prepared from polymer blends compared to those fabricated from pure polymers is associated with decreased MSC proliferation (data not shown). The proliferation rate of MSCs is known to slow down as they differentiate down various lineages<sup>70, 71</sup>, which further supports our finding that the blends do influence MSC function even in the absence of soluble differentiation factors. Whether the observed slowing down is associated with the same molecular pathways found in differentiating MSCs on plastic remains to be investigated<sup>70</sup>.

The MSCs used in this study were taken from the bone marrow of different patients so that the effects of different materials could be determined across a range of donors. Whilst this approach introduces greater biological variation, we believe that this is a more rigorous method than using multiple runs for the same patient as usually demonstrated in other studies. We have carefully taken into account the higher variation by using a robust statistical approach of ANOVA with a *post hoc* correction for multiple comparisons. MSCs exhibit heterogeneity at the transcriptome and proteome levels depending on their preparation<sup>72</sup>, source<sup>73</sup> and subset populations<sup>74</sup>. In order to assess how broad in scope the blends can drive MSC chondrogenic differentiation, future work will involve testing MSCs from other sources such as adipose tissue, umbilical cord and from pluripotent stem cell lines.

## 5. Conclusions

We have identified a specific blend of cellulose and silk polymers as a potent stimulator of MSC chondrogenic maturation independent of any chondrogenic growth factor stimulation. The use of ionic liquids as appropriate solvents enabled the preparation of a range of cellulose/silk membranes from which we were effectively able to screen the desired blend ratio to optimize the material's physical and chemical properties. The cellulose/silk blend identified here could be used both for *in vitro* tissue engineering and as an implantable device for stimulating endogenous stem cells to initiate cartilage repair. Future work will focus on preparing 3D scaffolds of the blend for preclinical studies.

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## Note:

The authors declare no competing financial interest.

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**Figure Captions**

**Figure 1.** Macroscopic and microscopic presentation of polymer membranes. Sheets of polymer membranes were cut into 8 mm discs (A). Scanning electron microscopy images showing cross sections of polymer membranes prepared from cellulose, and its blends with silk. B, D, F are low resolution images and C, E and G are high resolution images.

**Figure 2.** The chemical composition of regenerated cellulose, silk and cellulose/silk blends. Polymers and their blends were analyzed by FTIR and peaks of amide I and II were used to confirm the presence of silk components in the blends.

**Figure 3.** The influence of fibronectin coating on the blends' functional groups. Raman spectra of the neat cellulose (A-B), cellulose/silk 75:25 blend (C-D) and cellulose/silk 50:50 blends (E-F) which shows presence of functional groups associated with cellulose even after coating the surface of the membranes with fibronectin. The Raman spectra were collected according to the details given in the Experimental Section.

**Figure 4. Topography and surface roughness of blends.** Topography and surface roughness measurements of synthesized blend films were recorded by AFM imaging under ambient conditions. Two and ten micron height and phase images, 512 x 512 pixels in resolution, were collected. (A) Cellulose, (B) Cellulose/silk 75:25, and (C) Cellulose/silk 50:50.

**Figure 5.** Mechanical properties of pure and blended polymers. Samples were processed on a Textechno Favimat for stiffness and tensile strength according to the Experimental Section. (A) The stiffness (elasticity) of the cellulose and cellulose/silk blends. (B) The tensile strength of

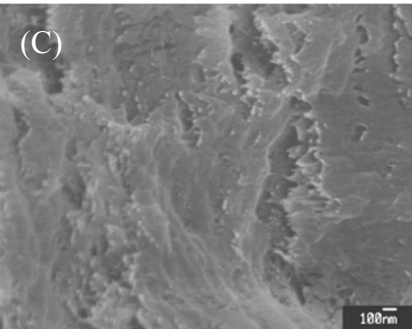
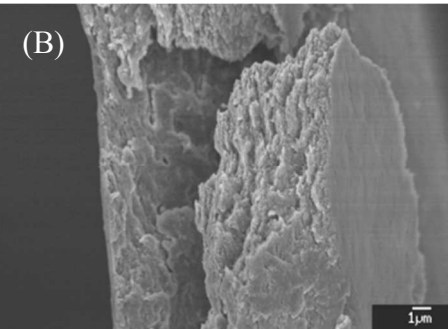
cellulose and cellulose/silk blends. \* $p < 0.05$  and \*\* $p < 0.01$  by ANOVA with a Bonferroni *post hoc* correction.

**Figure 6.** Viability of MSCs on polymer membranes. Cells were seeded on the scaffolds and stained with the LIVE/DEAD viability stain according to the Experimental Section (A-F). Green cells are live cells and red cells are dead cells due to the excitation of fluorescent dye, calcein AM at 490nm. The left column shows cells three days after seeding, and the right column shows cells seven days after seeding. Live cells (green) on plastic as positive control (G-H). Dead cells (red) treated with methanol (I).

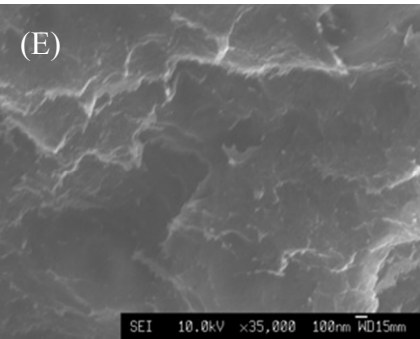
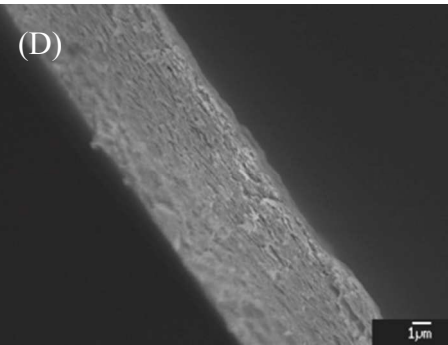
**Figure 7.** Chondrogenic gene expression of MSCs on polymer membranes. The RNA of MSCs ( $n=4$ ) grown on different polymers for 14 days was harvested and transcribed into cDNA for quantitative PCR analysis of the chondrogenic markers SOX9, aggrecan and type II collagen. Type I collagen was used as a marker of dedifferentiation. Relative gene expression was normalized to  $\beta$ -Actin as a housekeeping gene. Error bars denote SEM. \* $p < 0.05$  by Kruskal-Wallis with a Dunn *post hoc* correction.

**Figure 8.** Chondrogenic commitment of MSCs on polymer membranes. MSCs ( $n=4$ ) were grown on different polymers or plastic for 21 days in the presence or absence of the chondrogenic growth factor, TGF $\beta$  (A). Similarly, MSCs were incubated with or without adipogenic and osteogenic soluble supplements (B). Chondrogenic differentiation of MSCs was assessed by staining for aggrecan (red fluorescence antibody) and type II collagen (green fluorescence antibody). Adipogenic differentiation was assessed by staining fatty vacuoles with Oil Red O. Osteogenic differentiation was assessed by staining calcium with Alizarin Red.

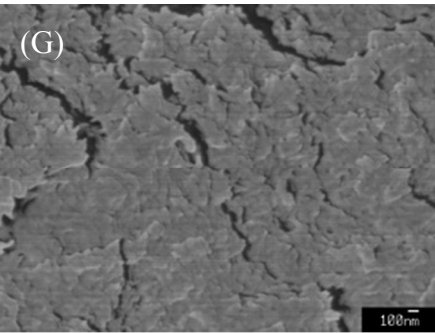
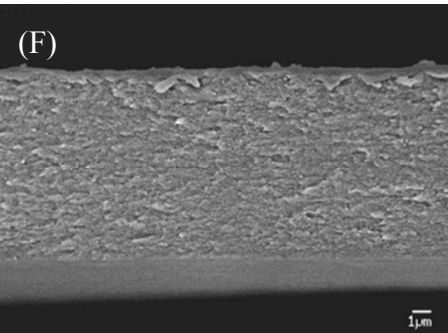
Figure 1



Cellulose



Cellulose/Silk  
75:25



Cellulose/Silk  
50:50

Figure 2

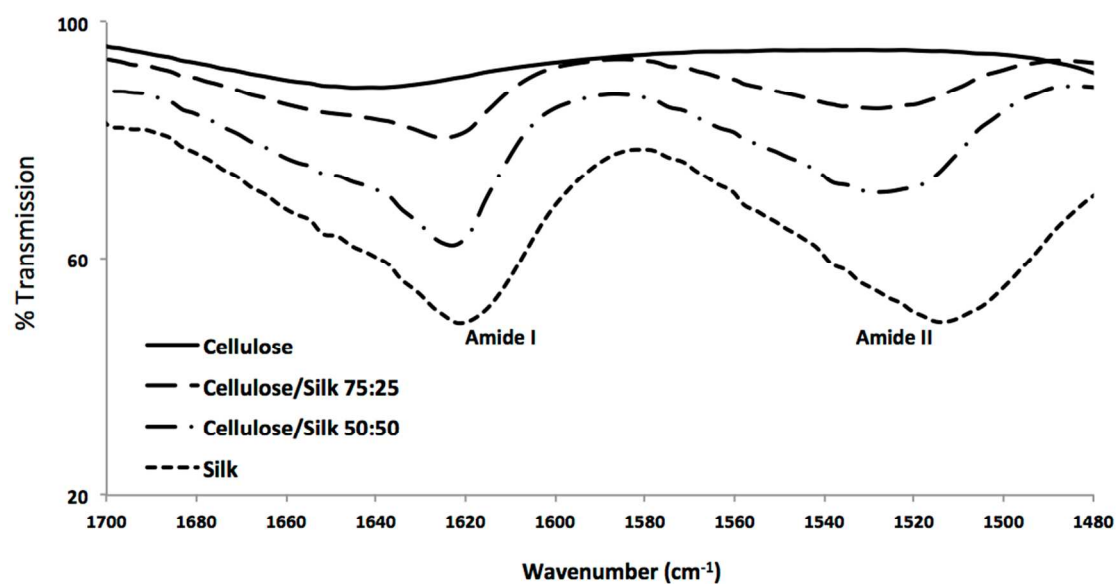


Figure 3

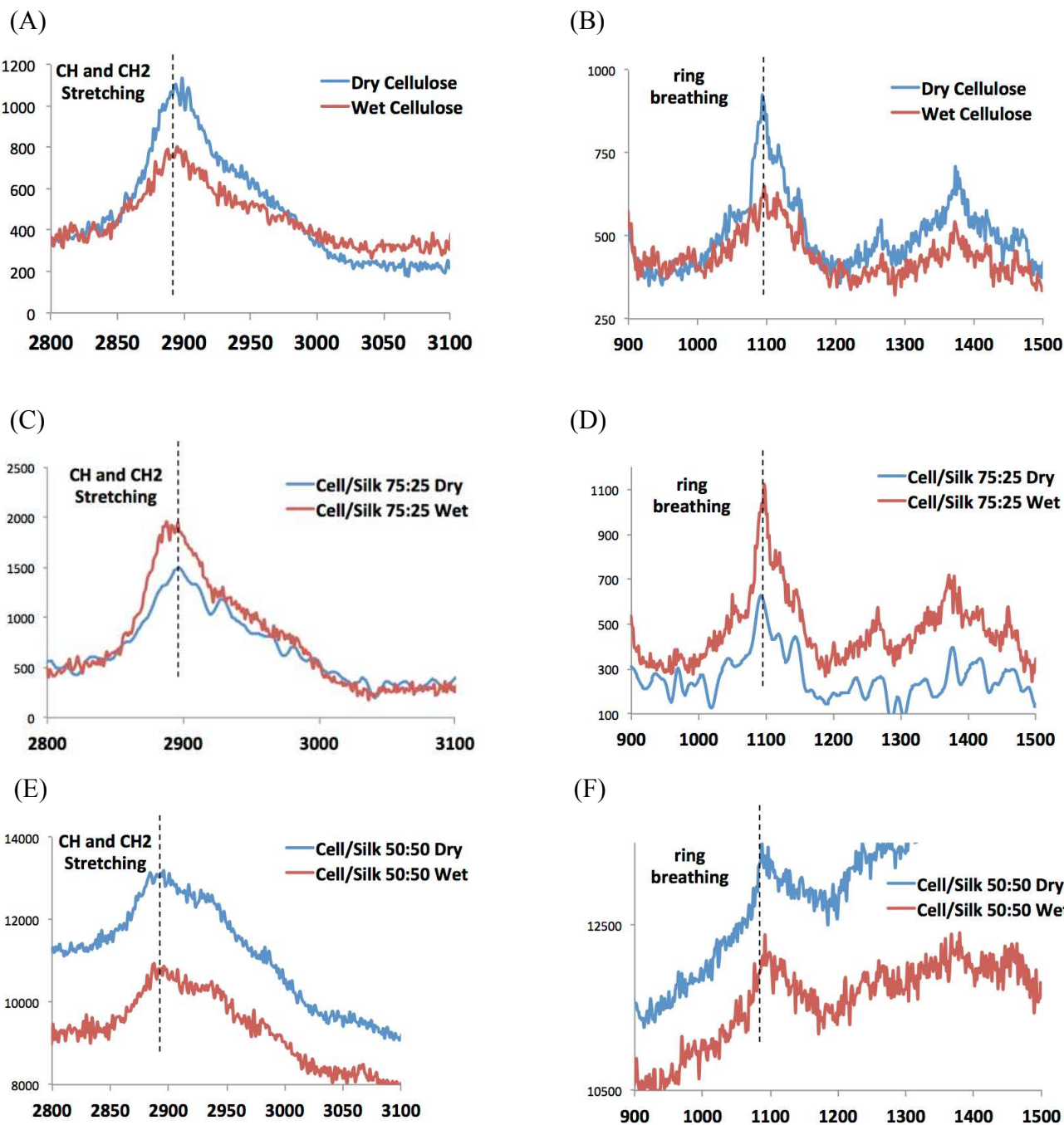




Figure 4

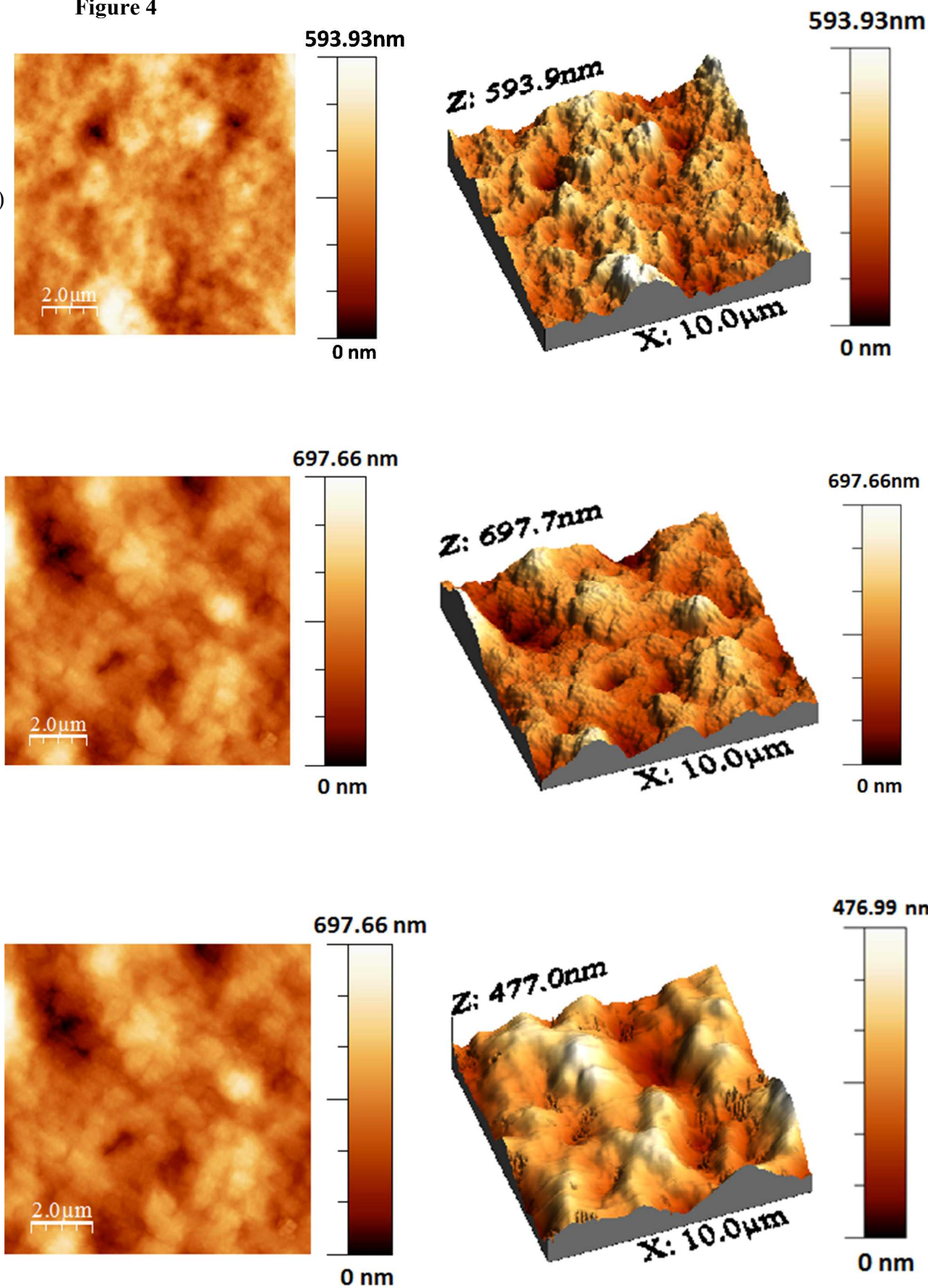




Figure 5

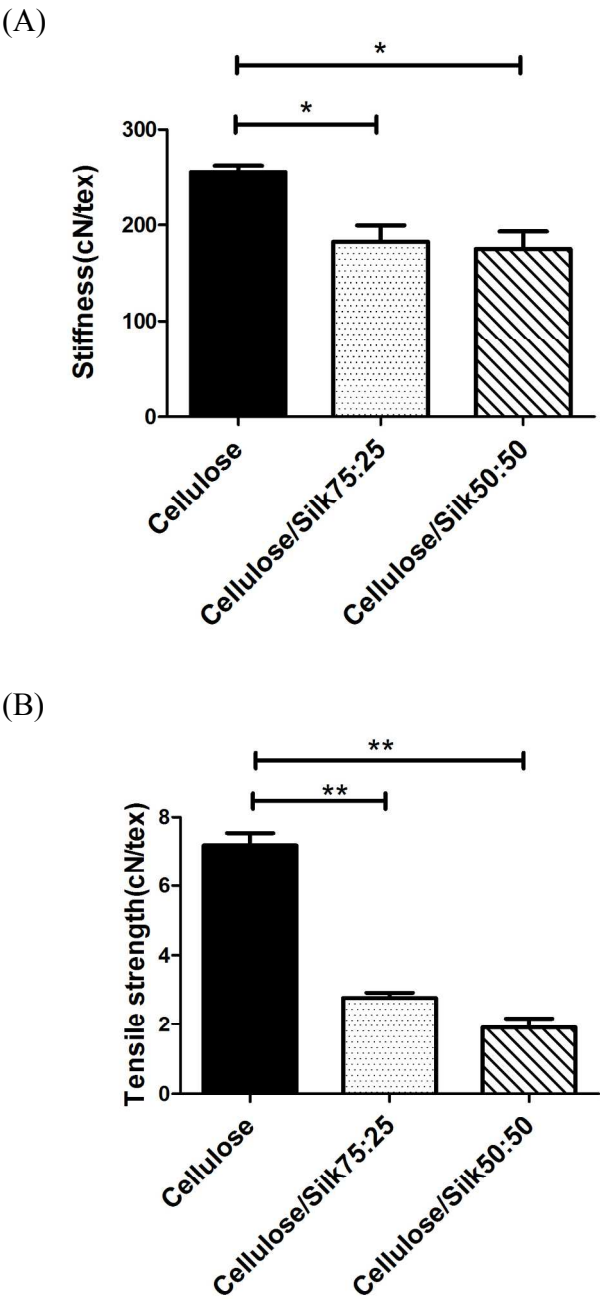


Figure 6

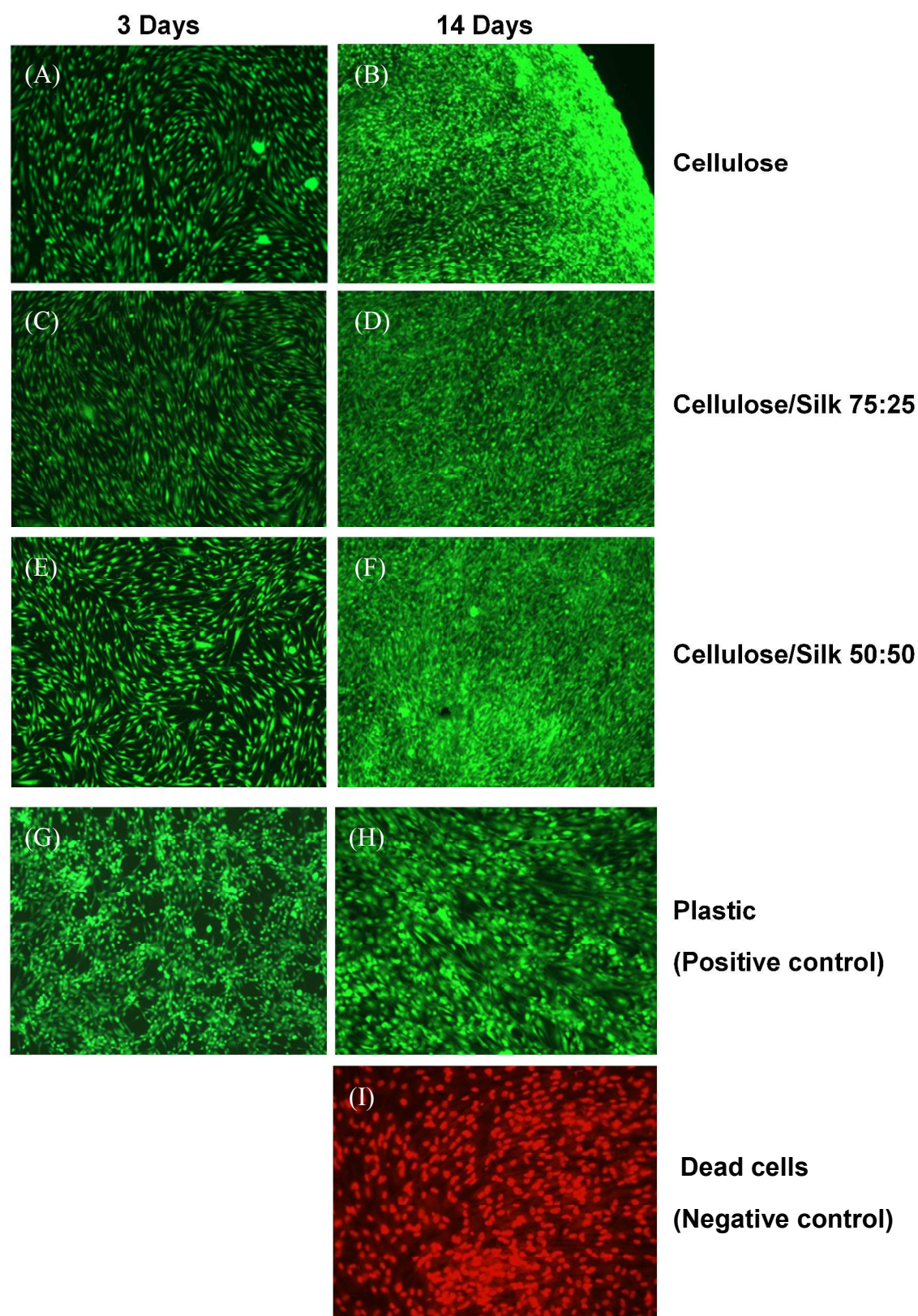
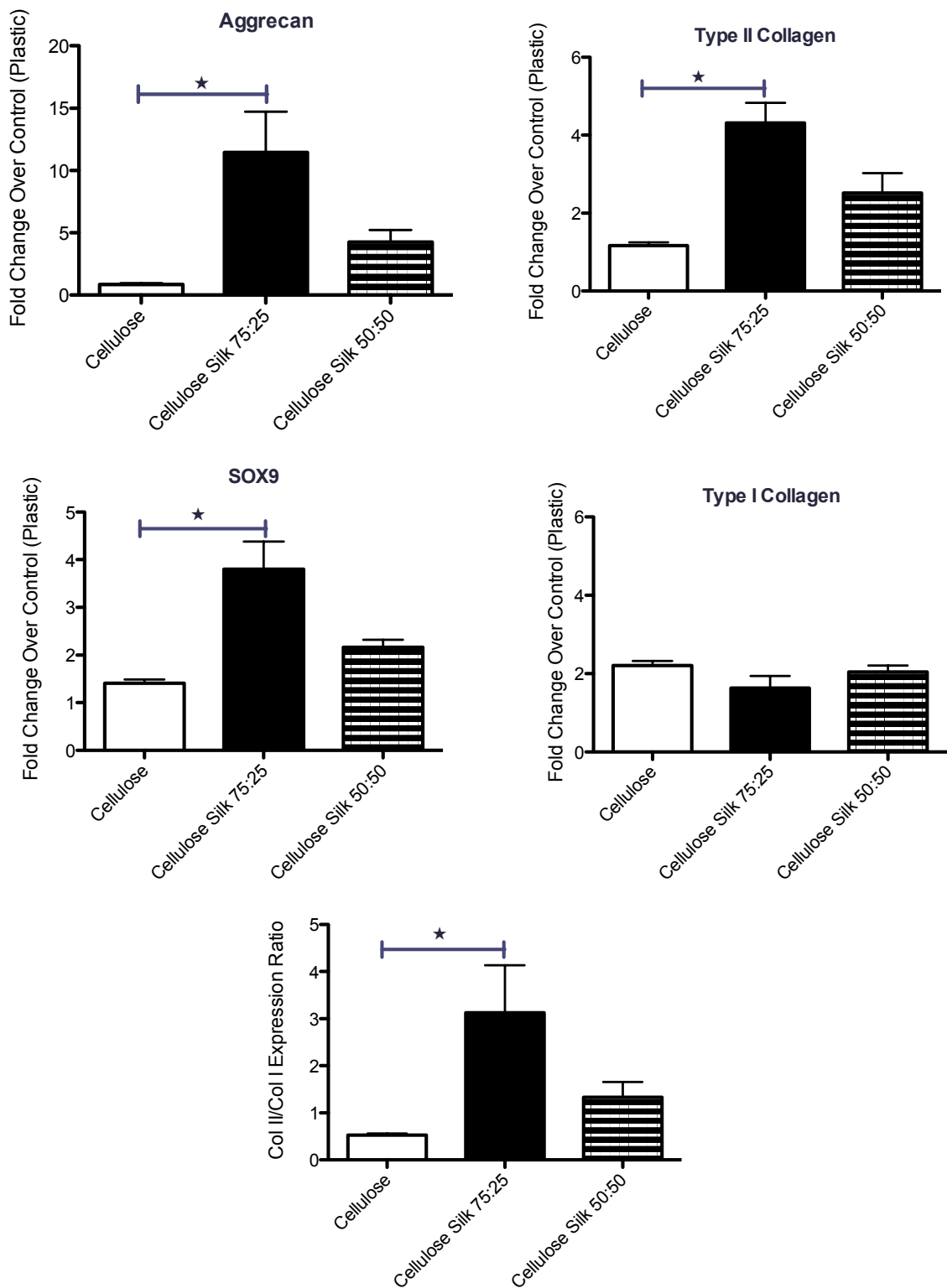
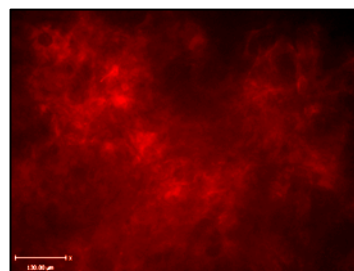
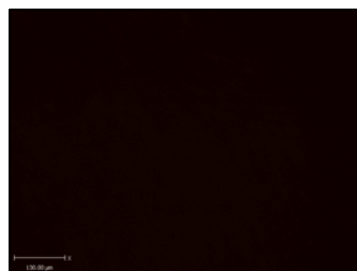
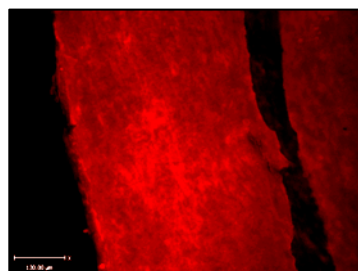
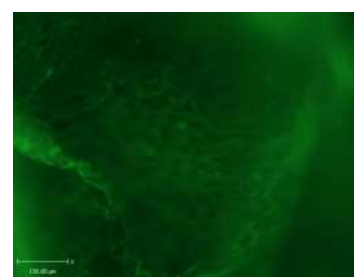
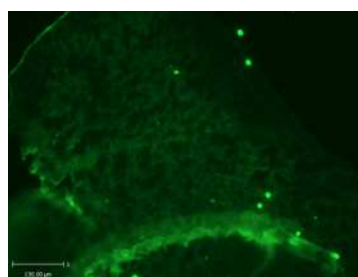


Figure 7



**Figure 8****(A)**Cellulose/silk 75:25  
without TGF $\beta$ MSCs on plastic  
without TGF $\beta$ MSCs on plastic  
with TGF $\beta$ 

Aggrecan



Type II Collagen

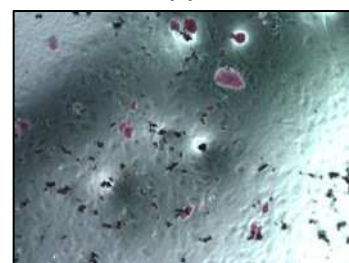
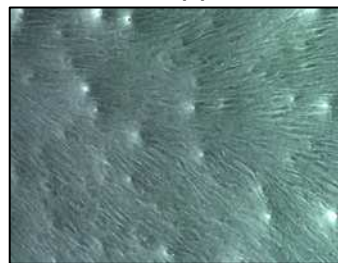
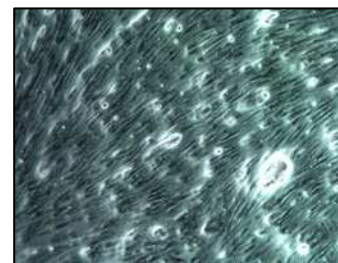
**(B)**Cellulose/Silk 75:25  
without supplementMSCs on plastic  
without supplementMSCs on plastic  
with supplement**Adipogenesis**  
(fatty vacuoles)**Osteogenesis**  
(calcium deposition)



Table of Contents Graphic

