Silk fibroin induces chondrogenic differentiation of canine adipose–derived multipotent mesenchymal stromal cells/mesenchymal stem cells

Metka Voga¹, Natasa Drnovsek², Sasa Novak² and Gregor Majdic¹,³

Abstract
Under appropriate culture conditions, mesenchymal stem cells (MSC), also called more properly multipotent mesenchymal stromal cells (MMSC), can be induced toward differentiation into different cell lineages. In order to guide stem cell fate within an environment resembling the stem cell niche, different biomaterials are being developed. In the present study, we used silk fibroin (SF) as a biomaterial supporting the growth of MMSC and studied its effect on chondrogenesis of canine adipose–derived MMSC (cADMMSC). Adipose tissue was collected from nine privately owned dogs. MMSC were cultured on SF films and SF scaffolds in a standard cell culture medium. Cell morphology was evaluated by scanning electron microscopy (SEM). Chondrogenic differentiation was evaluated by alcian blue staining and mRNA expression of collagen type 1, collagen type 2, Sox9, and Aggrecan genes. cADMMSC cultured on SF films and SF scaffolds stained positive using alcian blue. SEM images revealed nodule-like structures with matrix vesicles and fibers resembling chondrogenic nodules. Gene expression of chondrogenic markers Sox9 and Aggrecan were statistically significantly upregulated in cADMMSC cultured on SF films in comparison to negative control cADMMSC. This result suggests that chondrogenesis of cADMMSC could occur when cells were grown on SF films in a standard cell culture medium without specific culture conditions, which were previously considered necessary for induction of chondrogenic differentiation.

Keywords
Mesenchymal stem cells, multipotent mesenchymal stromal cells, dog, silk fibroin, chondrogenic differentiation

Date received: 14 September 2018; accepted: 11 February 2019

Introduction
Mesenchymal stem cells (MSC), more properly called multipotent mesenchymal stromal cells (MMSC), have received significant interest for their potential use in regenerative therapy in human and veterinary medicine due to their immunosuppressive and multilineage differentiation capabilities.¹ There are several established protocols for induction of MMSC differentiation in vitro, which are optimized for conventional culturing of MMSC in two-dimensional (2D) cell culture system with polystyrene vessels. Under appropriate culture conditions, differentiation of MMSC can be induced toward adipocytic, osteocytic, and chondrocytic lineages.²,³ Chondrogenesis can be induced with specific culture conditions such as high cell density, or by induction with different hormones and growth factors, particularly TFG-β.⁴⁻⁵ However, there are also several reports describing spontaneous differentiation of MMSC toward different lineages.⁶⁻⁹

¹Institute of Preclinical Sciences, Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia
²Department for Nanostructured Materials, Jozef Stefan Institute, Ljubljana, Slovenia
³Institute of Physiology, Medical School, University of Maribor, Maribor, Slovenia

Corresponding author:
Gregor Majdic, Institute of Preclinical Sciences, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia.
Email: gregor.majdic@vf.uni-lj.si

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (http://www.creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access page (https://us.sagepub.com/en-us/nam/open-access-at-sage).
Materials and methods

**Adipose tissue collection**

Subcutaneous adipose tissue was collected from nine privately owned dogs. Adipose tissue was individually collected during routine clinically indicated surgery at Small Animal Clinic of the Faculty of Veterinary Medicine in Ljubljana. All samples were randomly assigned to different experimental groups. All owners agreed with the collection of tissue and signed an informed consent. Since study was conducted on client-owned animals undergoing routine clinical procedure with owner’s approval to collect small piece of adipose tissue, no approval of ethical committee was needed according to Slovenian legislation and official opinion from The Administration of Republic of Slovenia for Food Safety, Veterinary and Plant protection, responsible for issuing ethical permits for animal experiments.

**Isolation and culture of cADMMSC**

Immediately after collection, adipose tissue was washed with Dulbecco’s Phosphate Buffered Saline (DPBS, Gibco, USA) and cut with a scalpel into small pieces. Adipose tissue was then incubated overnight at 37°C in Dulbecco-modified Eagle medium (DMEM, Gibco, USA) containing 0.1% collagenase type II (Sigma-Aldrich, DE). The digested tissue was centrifuged at 1600 r/min for 4 minutes, and the supernatant was discarded. Pellet of cells was resuspended in cell culture medium containing DMEM and 10% Fetal Bovine Serum (FBS, Gibco, USA). The cell suspension was plated into 6-well plates (TPP, Switzerland) and cultured at 37°C in a 5% CO₂ incubator. Cell culture medium was changed every 2–3 days. After 80%–90% confluence was reached, cells were trypsinized and multiplied by seeding 10⁴ cells per cm² into a larger (T25) cell culture flask. Cells in cell culture were maintained up to the fourth passage. After a sufficient number of cells was reached, cells were used for assessing chondrogenic differentiation. All cells used in the experiments were from the second, third or fourth passage, and cells were cultured for 2 or 3 days in each passage. All experiments were repeated five times for positive and negative controls and six times for cells cultured on SF films (both 1 week and 2 weeks).

**Multilineage differentiation potential of cADMMSC**

Differentiation potential was assessed by inducing cAD-MMSC differentiation into adipocytes, osteocytes, and chondrocytes. For the adipogenic and osteogenic differentiation 4 × 10⁴ cells were seeded in 12-well plates. When 90% confluency was reached, the cell culture medium was removed. Adipogenic (StemPro Adipogenesis Differentiation Kit, Gibco, USA) or osteogenic (StemPro
Osteogenesis Differentiation Kit, Gibco, USA) medium was added and changed every 2–3 days. Cell culture medium was added to the wells that served as negative controls. Adipogenic differentiation was analyzed with Oil-red-O staining (Sigma-Aldrich, DE) after 21 days of culturing. Osteogenic differentiation was analyzed with Alizarin Red S staining (Sigma-Aldrich, DE) after 14 days of culturing, which is shorter than recommended for human cells, but during our preliminary studies, we have established that with canine cells, 21 days of culture is too long period (cells start to detach) while majority of cells stain positive with Alizarine red already after 14 days of culturing. For the chondrogenic differentiation, micromass cultures were generated by seeding 5 µL droplets of 4 × 10^4 cells in the center wells of 12-well plate. After cultivating micromass cultures for 6 hours under high humidity conditions, chondrogenic medium (StemPro Chondrogenesis Differentiation Kit, Gibco, USA) was added to culture vessels. Regular cell culture medium was added to the wells that served as negative controls. Micromass cultures were incubated in 37°C incubator with 5% CO₂ and humid atmosphere. The medium was changed every 2–3 days. Chondrogenic differentiation was analyzed with alcin blue staining (Sigma-Aldrich, DE) after 14 days of culturing.

### Preparation of SF films and scaffolds

SF films and scaffolds were prepared following the procedure described by Rockwood et al. Briefly, Bombyx mori silk cocoons were cut in pieces and boiled for 30 minutes in 0.02 M solution of sodium carbonate (Na₂CO₃) to extract sericin. SF was rinsed in ultrapure water several times until the conductivity of water became constant and then dried overnight at 65°C. Degummed SF was dissolved in 9.3 M lithium bromide (LiBr) solution at 72°C for 3 hours and then subsequently dialyzed against 0.4 M LiBr for 48 hours. The molecular weight cut off of dialysis tubing cellulose membrane was 12–14 kDa. To eliminate impurities, the prepared solution was centrifuged at 20,000 r/min for 20 minutes. The concentration of SF solution was determined by the Bradford assay protocol based on the color change of Coomassie Brilliant Blue G-250 using Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA). The SF solution was added to the Bradford reagent and incubated for 5 minutes. The absorbance was measured at 595 nm. Two different concentrations of SF solution were used for the preparation of scaffolds and films. The concentration of the prepared solution was on average 8 mg/mL and was used for the preparation of scaffolds, whereas a higher concentration of SF solution, 12.5 mg/mL, was used for the preparation of films. Higher concentration was achieved using centrifugation through centrifugal filter units (Amicon Ultra–4 centrifugal filter unit, Merck, Cork, IE).

SF films were prepared by casting 300 µL of the SF solution (12.5 mg/mL) into the wells of 12-well plates with a subsequent overnight air-drying. Films were then incubated in 70% ethanol for 10 minutes. In the last step, films were thoroughly washed with PBS.

SF scaffolds were prepared by adding 300 µL of SF solution (8 mg/mL) into the wells of 48-well plate. SF solution in well plates was then frozen in liquid nitrogen and lyophilized at −50°C for 72 hours to sublime water and thus form porous scaffolds. After lyophilization, SF scaffolds were soaked in absolute ethanol overnight and then dried in a desiccator. Finally, scaffolds were thoroughly washed with PBS to remove any remaining ethanol.

### SF scaffold characterization

Porosity and the pore size distribution of the SF scaffolds were determined using a Pascal series mercury intrusion porosimeter (Thermo Scientific). The surface tension and the contact angle of the mercury were set to 0.485 and 140 mN/m, respectively.

Wettability of the SF film was evaluated by measuring water droplet contact areas of the curve fitted to the droplet image on a dry and wet SF film using the Contact Angle Instrument (First Ten Angstroms, Inc., USA, FTA1000 series). The measurement system consisted of a sample stage, vertically fitted Hamilton micro-syringe to place the water droplet on the sample and the camera mount–TV lens camera with Extension tube set 40 mm (Edmund optics, Japan). Images were captured and analyzed for contact areas using the FTA32 Video 2.0 software.

### Cultivation of cADMMSC on SF films and SF scaffolds

After a sufficient number of cells was obtained, cells were cultured in four different ways:

1. On SF films in cell culture medium for 7 and 14 days: 10^4 cells per cm² were seeded onto 12-well plate with wells coated with SF films.
2. On SF scaffolds in cell culture medium for 14 days: 9 × 5 µL droplets of 1 × 10^5 cells were seeded onto the bottom side of the SF scaffolds. During scaffold preparation, membrane-like portion of SF formed on the top of the scaffolds making the scaffold impassable for cells. Therefore, scaffolds were carefully lifted from the wells and turned upside down. Cells were then seeded onto the scaffolds.
3. On a standard polystyrene surface in chondrogenic medium for 14 days: cells were cultured as described above for a multilineage differentiation potential.
4. On a standard polystyrene surface in standard cell culture medium until 80%–90% confluency was reached.

Cell cultures were named accordingly (Table 1).
Alcian blue and DAPI staining

For alcian blue staining of SF film cADMMSC, positive control cADMMSC, and negative control cADMMSC, medium was removed from culture vessels. Wells were rinsed once with DPBS and cells were fixed with 4% paraformaldehyde solution for 1 hour. Following fixation, wells were rinsed three times with 0.1 N HCl. Next day wells were rinsed three times with 0.1 N HCl followed by DPBS to neutralize the acidity. Wells were examined under the light microscope. For comparison of alcian blue stained presumed chondrogenic nodules from positive control cADMMSC and SF film cADMMSC, some presumably chondrogenic nodules were immersed in tissue freezing medium (Leica Biosystems, Germany) and frozen in liquid nitrogen. The 2 µm thick cryosections were made with cryotome (Leica Biosystems), placed onto glass slides and visualized under the light microscope. For alcian blue staining of SF scaffold cADMMSC, the medium was removed from the wells. Whole SF scaffolds were immersed in tissue freezing medium (Leica Biosystems) and frozen in liquid nitrogen. The 18 µm thick cryosections were made with a cryotome (Leica Biosystems) and placed onto glass slides coated with 1% poly-L-lysine (Sigma-Aldrich, Germany). Slides were fixed following the protocol described elsewhere. Briefly, the medium was removed from culture vessels. Wells were rinsed once with DPBS and fixed with 2.5% glutaraldehyde solution (Sigma) overnight. After fixation, the samples were rinsed with 0.1 M cacodylate buffer (Sigma) at pH 7.4 for 1 hour with three changes, rinsed with distilled water for 1 min and then dehydrated in 10 minute steps in a series of ascending ethanol baths (25%, 50%, 75%, 95%, and 100%). Dehydrated samples were then immersed into hexamethyldisilazane bath (5 minutes, 100% HMDS) (Sigma), air-dried, mounted, and sputtered with gold.

Scanning electron microscopy (SEM) was also used for the estimation of film thickness and for pore size evaluation in SF scaffolds. Film thickness was measured from the cross-section image of SF film. Pore size was estimated by measuring the pore diameter in two directions, parallel and perpendicular to the surface of the scaffold. Diameters of all pores that were not touching the edge of an image were measured by ImageJ software on three low magnification images, where approximately a third of the scaffold is visible.

RNA isolation

RNA was isolated from SF film cADMMSC, positive control cADMMSC, and negative control cADMMSC. SF film and positive control cADMMSC were detached from the SF film and polystyrene surfaces using cell scraper. Negative control cADMMSC were detached by trypsinisation. The cell suspension was removed from the wells and
Voga et al.

centrifuged at 1600 r/min for 4 minutes. Pellet of cells was resuspended in DPBS and centrifuged again. Pellet of cells was then homogenized in 150 µL Trizol (ThermoFisher, USA) with a homogenizer (IKA T10 basic, Germany). Total RNA extraction was carried out according to the manufacturer’s protocol. The amount of extracted total RNA was measured by UV spectrophotometer (ThermoFisher) at 260/280 nm wavelength.

Reverse transcription quantitative polymerase chain reaction

Two-step reverse transcription quantitative polymerase chain reaction (RT qPCR) for SF film cADMMSC, positive control cADMMSC, and negative control cADMMSC was performed. First, 1 µg of total RNA of each specimen was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor (ThermoFisher) according to the manufacturer’s protocol. Negative reverse transcription controls were included in each PCR run. All reactions were conducted in a total volume of 20 µL. Conditions for reverse transcription were as suggested in the manufacturer’s protocol: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes. In the second step, relative quantification was performed using TaqMan Universal PCR Master Mix with UNG (ThermoFisher) and TaqMan gene expression assays Sox9, Col1A1, Col2A1, and Acan. TBP was used as a reference gene (Table 2; ThermoFisher). All qPCR amplifications were conducted in triplicates in a total volume of 20 µL. A 20 ng cDNA was used as a template. The amplification was carried out in 96-well plates with a Light Cycler 96 (Roche Life Science) using the following program: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds.

Statistical analyses

All statistical analyses were performed with NCSS software package (Kaysville, UT, USA).

Experiments with chondrogenic differentiation were repeated five times with cells from different dogs for negative and positive controls, and six times with cells from different dogs for cells growing on SF films. Three experiments with different cells were performed with SF scaffolds.

All RT qPCR experiments were run in triplicates. The efficiency corrected double delta Ct method was employed to normalize gene expression values. The expression levels of Col1A1, Sox9, Col2A1, and Acan in positive control cADMMSC and SF film cADMMSC were compared to the expression levels of Col1A1, Sox9, Col2A1, and Acan in negative control cADMMSC and results were analyzed by Kruskal–Wallis non-parametric test. Statistical significance was determined with p < 0.05.

Results

Culturing and multilineage differentiation of negative control cADMMSC

Adipose tissue was successfully collected from all animals. Under the light microscopy, cells with fibroblast-like morphology were observed in all samples the day after plating. Using SEM, negative control cADMMSC appeared flat with spread morphology and wide extensions (Figure 1).

Morphology and alcian blue analysis of positive SF film cADMMSC

SF film cADMMSC were cultured in normal culture medium on SF films. SF films prepared from SF solution with a concentration of 12.5 mg/mL were transparent, with diameter of 21 mm, and the approximate dry film thickness

Table 2. Symbols, names, and assay identification numbers of genes of interest and their role in MSC chondrogenesis.

| Gene symbol | Gene name                          | Assay ID          | Gene role in chondrogenesis                              |
|-------------|------------------------------------|-------------------|----------------------------------------------------------|
| Sox9        | SRY (sex determining region Y)-box9| cf02625134_g1     | The first transcription factor, essential for chondrocyte differentiation and cartilage formation40 |
| Col1A1      | Collagen type I, alpha 1           | Cf01076765_m1     | Most abundant in preaggregate cells5                    |
| Col2A1      | Collagen type II, alpha 1          | cf02622868_m1     | Cartilage specific marker gene40                         |
| Acan        | Aggrecan                           | Cf02674826_m1     | Cartilage specific marker gene41                         |
| TBP         | TATA box binding protein           | cf02637231_m1     | Reference gene                                           |

MSC, mesenchymal stem cells.
of 80 µm. The surface of the film appears smooth and dense, some roughness is visible only on a submicron level caused by nanosized pores that are assumed to form during drying of the film (Figure 3). The contact angle of a water droplet on a dry SF film is 70°, indicating a hydrophobic surface. After washing the film with PBS, the surface becomes hydrophilic with a water contact angle of 0°.

SF film cADMMSC were cultured in normal cell culture media on SF films. SF films were transparent, with diameter of 21 mm, approximate thickness of 900 µm and average fibroin density of 12.5 mg/mL. Cells successfully adhered onto SF film surface. In comparison to negative control cADMMSC (Figure 1), SF film cADMMSC displayed less spindle-shaped morphology (Figure 4(a)). A tendency toward cell grouping was observed. The second day after seeding, nodule-like structures began to form (Figure 4(b)).

Positive control cADMMSC were cultured in the chondrogenic medium on a standard polystyrene surface for 14 days. Cells changed their morphology from adherent monolayer spindle-shaped cells to layered nodule-like cell clusters. Nodules that morphologically resembled chondrogenic nodules tended to form connections among each other and stained positive with alcian blue (Figure 5(a)). SEM images of positive control cADMMSC confirmed chondrogenic nodule-like structures with many extracellular matrix vesicles (Figure 5(d)).

One to three days after culturing cADMMSC on SF films, chondrogenic-like nodules appeared (Figure 5(b) and (c)), similar to those formed in positive control cADMMSC (Figure 5(a)), with indistinguishable intensity of alcian blue staining. No apparent difference in alcian blue staining between SF film cADMMSC cultured for 7 and 14 days was detected (Figure 5(b) and (c)). SEM analysis of SF film cADMMSC revealed chondrogenic nodule-like structures (Figure 5(e) and (f)), comparable to those formed in positive control cADMMSC (5d). Many extracellular matrix vesicles were also observed. Nodule-like structures in SF film cADMMSC cultured for 14 days (Figure 5(f)) appeared to be more defined in comparison to those cultured for 7 days (Figure 5(e)).

Figure 1. SEM image of negative control cADMMSC that appear flat with spread morphology and wide extensions.

Figure 2. Differentiation potential of cADMMSC. Adipogenic potential of cADMMSC is indicated by red intracellular lipid droplets using Oil-red-O (a). In osteogenic differentiation, mineral deposits in extracellular matrix are stained red using Alizarin-red-S (b). Chondrogenic differentiation is indicated by the formation of chondrogenic nodules that stain blue with alcian blue (c). Respective negative controls are shown at the bottom (d, e, f).
Cryosections of alcian blue stained chondrogenic-like nodules from positive control cADMMSC revealed the correct round shape of a nodule, enclosed within a capsule-like structure. Nodule fibers appeared thick and homogenously arranged (Figure 6(a)). Cryosections of alcian blue stained chondrogenic-like nodules from SF film cADMMSC revealed more irregularly shaped nodules without an apparent capsule compared to cryosections of positive control chondrogenic nodule. Nodule fibers were thinner and organized into smaller separate circles formed inside of a nodule (Figure 6(b)).

**Gene expression of chondrogenic markers**

The mRNA expression of Col1A1 was similar in all four samples (Figure 7(a), N=5 for positive and negative control, and 6 for SF films after 1 and 2 weeks) and Acan (Figure 7(d), N=3 for positive control, 4 for negative control, and 6 for SF films after 1 and 2 weeks) was statistically significantly higher in positive control cADMMSC and SF film cADMMSC in comparison to negative control cADMMSC (p < 0.05 for Sox9 and p < 0.01 for Acan). The expression level of Col2A1 (N=4 for positive and negative control, and 6 for SF films after 1 and 2 weeks) was not statistically significantly different between negative and positive control cADMMSC and SF film cADMMSC (Figure 7(b)).

**SF scaffold characteristics**

The volume of the scaffolds was 0.3 mL with an approximate thickness of SF scaffolds 5.2 mm. Light microscopy images of unseeded cryosections of scaffold showed a porous matrix with interconnected elongated pores (Figure 8(a) and (b)). Scaffold porosity was 92.7%, of which
84.6% represents open, and 8.1% closed porosity. The mean pore opening diameter measured as a function of pressure from the mercury intrusion measurements was 50 µm, with a minimum and maximum at 0.004 and 107 µm, respectively. SEM images of nonseeded SF scaffold revealed a porous matrix with a broad distribution of pore sizes, where the bottom side of a scaffold had larger pores (Figure 8(c)). Mean pore diameter measured on SEM images in the elongated direction was $314 \pm 296$ µm and $58 \pm 55$ µm in the direction parallel to the surface.

**Morphology and alcian blue analysis of positive, SF scaffold cADMMSC**

SF scaffold cADMMSC were cultured in culture medium in SF scaffolds. Cell migration into the scaffold was analyzed...
by a fluorescent microscope with DAPI staining (Figure 9(c) and (d)) and SEM (Figure 10). Based on the presence of the cells in all cryosections, it was concluded that SF scaffold structure allows penetration of cells inside the scaffold, although more cells remained on the surface of the scaffold (Figure 9). Nevertheless, some cells migrated throughout the scaffold and successfully attached to the pore walls. Positive alcian blue staining of cells was confirmed on the surface and within the scaffold (Figure 9(a) and (b)). Chondrogenic nodules were observed only occasionally close to the surface of the scaffold (Figure 9(b)). Mostly, amorphous layers of cells were observed, but these also stained positive with alcian blue (Figure 9(a)).

SEM analysis of SF scaffold cADMMSC confirmed homogeneous distribution of cells throughout the scaffold and their attachment to the pore walls. A dense network of...
fibers was formed, and numerous extracellular matrix vesicles were observed (Figure 10(a) and (b)—scaffold with cells at different magnifications).

**Discussion**

In attempts to mimic the native extracellular matrix, numerous studies are focusing on culturing MMSC in a 3D culture system, which better imitates their natural environment. Although several biomaterials have been shown to promote differentiation of MMSC toward different lineages, there are no reports about SF inducing differentiation of MMSC. In the present study, we demonstrate that cADMMSC presumably undergo chondrogenic differentiation when grown on SF films and SF scaffolds in a standard cell culture medium. cADMMSC appeared to follow chondrogenesis without chondrogenic-specific conditions such as high cell density or stimulation with TGF-β. Morphology of cells observed under the microscope, positive alcian blue staining of the SF film cADMMSC and SF scaffold cADMMSC, and upregulation of Sox9 and Aggrecan mRNA expression in cADMMSC on SF films suggest that cells underwent chondrogenic differentiation. Chondrogenesis of cADMMSC cultured on SF films was also suggested by similar chondrogenic-like nodule morphology, and a tendency to form interchondrogenic nodule connections as it was observed in positive control cADMMSC. In comparison to cells cultured on SF films, cADMMSC cultured inside the SF scaffolds formed chondrogenic-like nodules only in parts of the scaffold. Elsewhere, amorphous layers of cells were present, but, interestingly, both nodules and amorphous layers of cells inside the SF scaffolds stained positive with alcian blue suggesting at least partial induction of chondrogenesis. Comparison of SEM images between positive control cADMMSC, SF film cADMMSC, and SF scaffold cADMMSC showed the presence of cell nodules that resembled chondrogenic nodules. In all three groups of cells, many extracellular matrix vesicles were present and abundant extracellular matrix formed which appeared as chondrogenic-like nodule structures. SEM images of SF scaffold cADMMSC

![Figure 9. SF scaffold cADMMSC. In upper row, alcian blue staining of scaffold cryosections is shown. In lower rows, the same images are shown under fluorescence, where cell nuclei were stained with DAPI (white arrowheads). In parts of the scaffold surface, chondrogenic nodule-like structures (black arrow) were present (b) and cell density in these structures was very dense (white arrowheads—cell nuclei stained with DAPI in panel (d)). Inside the scaffold (panels a and c) cells were present, but at lower density in comparison to surface (white arrows—cell nuclei stained with DAPI in (c) and (d)) and they did not form chondrogenic nodules, although they stained positive with alcian blue (arrow, a). SF structure is marked with red arrowheads in panels a and b.](image-url)
also revealed numerous fibers formed by the cells, which suggest possible formation of collagen.

It has been previously demonstrated that in vitro initial cell aggregates, cultured on polystyrene surface under specific culture conditions, contain Col1A1. Within 2–3 weeks of chondrogenic differentiation, MMSC undergo chondrogenic differentiation and start to produce abundant extracellular matrix composed of Col2A1. Differentiation of MMSC is characterized by a decrease in proliferation and upregulation of lineage-specific genes. TGF-β is known to induce chondrogenesis by activating SMAD signaling pathway and upregulating chondrogenic genes such as Sox9. The latter has been identified as the main transcriptional regulator of chondrogenic specific markers, namely, type II collagen. Another marker of chondrogenic differentiation is Aggrecan, an essential component of mature cartilage, whose expression also increases during chondrogenic differentiation of MMSC. In our study, the expression of Col1A1 and cartilage-specific markers Sox9, Aggrecan, and Col2A1 were determined by RT qPCR in cells grown on SF films and positive and negative controls. mRNA expression of Col1A1 and Col2A1 was not significantly different between positive and negative controls cADMMSC and SF film cADMMSC. However, both Aggrecan and Sox9 mRNA expression was statistically significantly upregulated in positive control cADMMSC and SF film cADMMSC. This suggests that chondrogenesis was indeed initiated also at the molecular level. Currently, it is difficult to speculate why we did not also detect increased expression of Col2A1. However, it has to be noted that mRNA expression of Col2A1 was very low and perhaps, the time of chondrogenesis was too short to induce also Col2A1, a marker of mature chondrocytes. The RT qPCR results of our study thus indicate that chondrogenic differentiation of SF film cADMMSC is comparable to positive control cADMMSC. Based on the upregulation of Sox9 and Aggrecan in both positive control and SF film cADMMSC, we concluded that the initial stages of chondrogenesis took place, but it might take longer than 14 days for Col2A1 to be significantly upregulated. Due to protein overload and lower number of cells when isolating RNA from cells with proteins from the scaffold, we were unable to purify enough RNA perform RT qPCR from SF scaffold cADMMSC to confirm mRNA expression results also with cells grown in SF scaffolds.

Mechanisms behind presumed chondrogenic differentiation of cADMMSC on SF in our study are not yet known. In line with the high cell density as one of the

Figure 10. SEM images of cADMMSC grown on SF scaffold: (a) cells attached to the pore wall. Numerous matrix vesicles and fibers are seen on top of the cells; (b) cell inside the scaffold pore at higher magnification. Matrix vesicles are protruding from the cell; (c, d) Same cells marked with color.
ADMMSC were used, which are rarely studied. There are not typic changes under the same conditions. In our study, bone marrow–derived MMSC did not undergo similar phenotype dishes in a standard culture medium. However, rat into both chondrocytic and osteocytic cells on plastic cultured on SF films but also on architecturally different 3D SF scaffolds. Different parameters used in each study such as donor species, tissue source of MMSC, culture media composition, and culturing methods makes difficult any direct comparisons of these studies. Future studies are therefore necessary to determine which factors might contribute to the mechanisms lying behind chondrogenic differentiation of MMSC in the absence of special growth factors and/or high cell density seeding. Different parameters used in each study such as donor species, tissue source of MMSC, culture media composition, and culturing methods makes difficult any direct comparisons of these studies. Future studies are therefore necessary to determine which factors might contribute to the mechanisms lying behind chondrogenic differentiation of MMSC in the absence of special growth factors.

Results of our study show that SF could be considered as a promising biomaterial not only for culturing cells but also for the induction of controlled chondrogenesis of MMSC. None of the previous studies using SF has shown its ability to guide MMSC toward chondrogenic differentiation, so this might be a species-specific effect, as our study was the first study to examine the growth of canine MMSC on SF. This effect of SF on MMSC in our study, therefore, represents a basis for further studies aiming to understand the key factors and mechanisms responsible for induction of differentiation of MMSC into chondrocytes.

Data availability
The raw data and processed data required to reproduce these findings are available from the authors upon request.
Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grant P4-0053 from Slovenian research agency and Metka Voga is supported by a graduate fellowship from the Slovenian research agency.

ORCID iD
Gregor Majdic https://orcid.org/0000-0001-9620-2683

References
1. Lee DE, Ayoub N and Agrawal DK. Mesenchymal stem cells and cutaneous wound healing: novel methods to increase cell delivery and therapeutic efficacy. Stem Cell Res Ther 2016; 7: 37.
2. Dennis JE, Merriam A, Awadallah A, et al. A quadripotent mesenchymal progenitor cell isolated from the marrow of an adult mouse. J Bone Miner Res 1999; 14(5): 700–709.
3. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284(5411): 143–147.
4. Cancedda R, Descalzi Cancedda F and Castagnola P. Chondrogenic differentiation. Int Rev Cytol 1995; 159: 265–358.
5. Johnstone B, Hering TM, Caplan AI, et al. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 1998; 238: 265–272.
6. Bosnakovski D, Mizuno M, Kim G, et al. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet culture system. Exp Hematol 2004; 32(5): 502–509.
7. De Bari C, Dell’Accio F and Luyten FP. Human perios- teum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. Arthritis Rheum 2001; 44(1): 85–95.
8. Dudakovic AD, Camilleri E, Riester SM, et al. High-resolution molecular validation of self-renewal and spontaneous differentiation in adipose-tissue derived human mesenchymal stem cells cultured in human platelet lysate. J Cell Biochem 2015; 115: 1816–1828.
9. Naruse K, Urabe K, Mukaida T, et al. Spontaneous differentiation of mesenchymal stem cells obtained from fetal rat circulation. Bone 2004; 35(4): 850–858.
10. Martino S, D’Angelo F, Armentano I, et al. Stem cell-biocompatibility interactions for regenerative medicine. Biotechnol Adv 2012; 30(1): 338–351.
11. Robey PG. Stem cells in tissue engineering. In: Atala A and Lanza R (eds) Handbook of stem cells. Amsterdam: Elsevier, 2013, pp. 5–12.
12. Du J, Chen X, Liang X, et al. Integrin activation and internalization on soft ECM as a mechanism of induction of stem cell differentiation by ECM elasticity. Proc Natl Acad Sci USA 2011; 108(23): 9466–9471.
13. Park JS, Chu JS, Tsou AD, et al. The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta. Biomaterials 2011; 32(16): 3921–3930.
14. Topal AE, Tekinay AB, Guler MO, et al. Mechanical properties of differentiating stem cells on peptide nanofibers. Biophys J 2016; 110: 624a.
15. Benoit DS, Schwartz MP, Durney AR, et al. Small molecule functional groups for the controlled differentiation of human mesenchymal stem cells encapsulated in poly (ethylene glycol) hydrogels. Nat Mater 2008; 7: 816–823.
16. Curran JM, Chen R and Hunt JA. The guidance of human mesenchymal stem cell differentiation in vitro by controlled modifications to the cell substrate. Biomaterials 2006; 27(27): 4783–4793.
17. Ren YJ, Zhang H, Huang H, et al. In vitro behavior of neural stem cells in response to different chemical functional groups. Biomaterials 2009; 30(6): 1036–1044.
18. D’Angelo F, Armentano I, Mattioli S, et al. Micropatterned hydrogenated amorphous carbon guides mesenchymal stem cells towards neuronal differentiation. Eur Cell Mater 2010; 20: 231–244.
19. Guvendiren M and Burdick JA. The control of stem cell morphology and differentiation by hydrogel surface wrinkles. Biomaterials 2010; 31(25): 6511–6518.
20. Kilian KA, Bugarija B, Lahn BT, et al. Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci USA 2010; 107(11): 4872–4877.
21. Xu X, Kratz K, Wang W, et al. Cultivation and spontaneous differentiation of rat bone marrow-derived mesenchymal stem cells on polymeric surfaces. Clin Hemorheol Microcirc 2013; 55(1): 143–156.
22. Zhao J, Zhang Z, Wang S, et al. Apatite-coated silk fibroin scaffolds to healing mandibular border defects in canines. Bone 2009; 45(3): 517–527.
23. Lee JH, Shin YC, Jin OS, et al. Reduced graphene oxide-coated hydroxyapatite composites stimulate spontaneous osteogenic differentiation of human mesenchymal stem cells. Nanoscale 2012; 7: 11642–11651.
24. Sonomoto K, Yamaoka K, Kaneko H, et al. Spontaneous differentiation of human mesenchymal stem cells on poly-lactic-co-glycolic acid nano-fiber scaffold. PLoS ONE 2016; 11(4): e0153231.
25. Zhang N, Xiao Q-R, Man X-Y, et al. Spontaneous osteogenic differentiation of mesenchymal stem cells on electrospun nanofibrous scaffolds. RSC Adv 2016; 6: 22144–22152.
26. Kundu B, Rajkhowa R, Kundu SC, et al. Silk fibroin biomaterials for tissue regenerations. Adv Drug Deliv Rev 2013; 65(4): 457–470.
27. Altman GH, Diaz F, Jakuba C, et al. Silk-based biomaterials. Biomaterials 2003; 24: 401–416.
28. Yuec T, Lovett ML and Kaplan DL. Silk-based biomaterials for sustained drug delivery. J Control Release 2014; 190: 381–397.
29. Rockwood DN, Preda RC, Yücel T, et al. Materials fabrication from Bombyx mori silk fibroin danielle. Nat Protoc 2013; 6: 1612–1631.
30. Bai S, Han H, Huang X, et al. Silk scaffolds with tunable mechanical capability for cell differentiation. Acta Biomater 2015; 20: 22–31.
31. Chen J, Altman GH, Karageorgiou V, et al. Human bone marrow stromal cell and ligament fibroblast responses on RGD-modified silk fibers. *J Biomed Mater Res A* 2003; 67(2): 559–570.

32. Jaipaew J, Wangkulangkul P, Meesane J, et al. Mimicked cartilage scaffolds of silk fibroin/hyaluronic acid with stem cells for osteoarthritis surgery: morphological, mechanical, and physical clues. *Mater Sci Eng C Mater Biol Appl* 2016; 64: 173–182.

33. Meinel L, Hofmann S, Karageorgiou V, et al. Engineering cartilage-like tissue using human mesenchymal stem cells and silk protein scaffolds. *Biotechnol Bioeng* 2004; 88(3): 379–391.

34. Rodriguez-Lozano FJ, Garcia-Bernal D, Aznar-Cervantes S, et al. Effects of composite films of silk fibroin and graphene oxide on the proliferation, cell viability and mesenchymal phenotype of periodontal ligament stem cells. *J Mater Sci Mater Med* 2014; 25(12): 2731–2741.

35. Zhang Y, Fan W, Ma Z, et al. The effects of pore architecture in silk fibroin scaffolds on the growth and differentiation of mesenchymal stem cells expressing BMP7. *Acta Biomater* 2010; 6: 3021–3028.

36. Rockwood DN, Preda RC, Yucel T, et al. Materials fabrication from *Bombyx mori* silk fibroin. *Nat Protoc* 2011; 6(10): 1612–1631.

37. Bradford MM and Williams WL. New, rapid, sensitive method for protein determination. *Fed Proc* 1976; 35: 274.

38. Bergmans L, Moisiadis P, Van Meerbeek B, et al. Microscopic observation of bacteria: review highlighting the use of environmental SEM. *Int Endod J* 2005; 38(11): 775–788.

39. Pintens V, Masson C, Merckx R, et al. The role of sigma(B) in persistence of *Staphylococcus epidermidis* foreign body infection. *Microbiol-Sgm* 2008; 154: 2827–2836.

40. Bi WM, Deng JM, Zhang ZP, et al. Sox9 is required for cartilage formation. *Nat Genet* 1999; 22(1): 85–89.

41. Goldring MB. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Ther Adv Musculoskelet Dis* 2012; 4(4): 269–285.

42. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; 29(9): e45.

43. Stein GS, Lian JB and Owen TA. Relationship of cell-growth to the regulation of tissue-specific gene-expression during osteoblast differentiation. *FASEB J* 1990; 4(13): 3111–3123.

44. Xu J, Wang W, Ludeman M, et al. Chondrogenic differentiation of human mesenchymal stem cells in three-dimensional alginate gels. *Tissue Eng Part A* 2008; 14(5): 667–680.

45. Fortier LA, Nixon AJ, Williams J, et al. Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells. *Am J Vet Res* 1998; 59(9): 1182–1187.