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Fibrochondrogenic potential of synoviocytes from osteoarthritic and normal joints cultured as tensioned bioscaffolds for meniscal tissue engineering in dogs

Meniscal tears are a common cause of stifle lameness in dogs. Use of autologous synoviocytes from the affected stifle is an attractive cell source for tissue engineering replacement fibrocartilage. However, the diseased state of these cells may impede in vitro fibrocartilage formation. Synoviocytes from 12 osteoarthritic ("oaTSB") and 6 normal joints ("nTSB") were cultured as tensioned bioscaffolds and compared for their ability to synthesize fibrocartilage sheets. Gene expression of collagens type I and II were higher and expression of interleukin-6 was lower in oaTSB versus nTSB. Compared with nTSB, oaTSB had more glycosaminoglycan and alpha smooth muscle staining and less collagen I and II staining on histologic analysis, whereas collagen and glycosaminoglycan quantities were similar. In conclusion, osteoarthritic joint-origin synoviocytes can produce extracellular matrix components of meniscal fibrocartilage at similar levels to normal joint-origin synoviocytes, which makes them a potential cell source for canine meniscal tissue engineering.

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17 1. Introduction:

18 Meniscal injury is a common cause of lameness and pain of the dog. Due to the virtually
19 absent healing response in the majority of the meniscus, injured meniscal tissue is commonly
20 removed to relieve the clinical signs of lameness, joint locking, and painful popping.
21 Unfortunately, partial meniscectomy hastens the development of secondary arthritis (Berjon et
22 al., 1991; Connor et al., 2009; Cox et al., 1975) and thus patient lameness.

23 Tissue engineering methods are being investigated to address this challenge of meniscal
24 injury and loss. One of the great obstacles to achieving the reality of tissue engineered menisci is
25 determination of an ideal cell source for *in vitro* culture and extracellular matrix (ECM)
26 formation. Because cells cannot be synthesized *de novo*, they must be harvested autologously, or
27 obtained from living or deceased tissue donors. When determining ideal cell sources for tissue
28 engineering, location of the source tissue, quantity of donor tissue available for harvest, and
29 ability to harvest the cells in a minimally invasive fashion must be considered. Autologous cells
30 are particularly attractive because they have a low potential for infectious disease transmission
31 (Pessina et al., 2008) and immunogenic tissue rejection (Hamlet et al., 1997; Ochi et al., 1995;
32 Rodeo et al., 2000).

33 While producing normal, healthy menisci *in vitro* is the ultimate goal of tissue
34 engineering, use of normal meniscal cells from a healthy donor site would cause irreversible
35 patient harm and thus is a poor choice for meniscal tissue engineering. Mesenchymal stem cells
36 were recently identified in normal joint-origin canine synovium (Zhang et al., 2013), which could
37 be used towards *in vitro* meniscal fibrochondrogenesis. When cultured in monolayer, cells
38 obtained from the synovial membrane of normal joints are primarily positive for CD90 (marker
39 for stemness), CD29 (β - integrin), CD44 (hyaluronic acid receptor) and negative for markers of

40 hematopoietic progenitors (CD34) and leukocyte antigens (CD45; Zhang et al.2013). These cells
41 are also able to undergo chondrogenesis when cultured in pellet form (Zhang et al.2013).
42 However, clinical use of normal autologous synoviocytes as a tissue engineering cell source
43 would require surgery on another unaltered joint within the patient's body.

44 Autologous, osteoarthritic joint-origin synovium has been investigated as a cell source for
45 fibrocartilage tissue engineering in dogs, because of its abundance and ease of harvest during
46 clinically required surgical procedures (Warnock et al., 2012). In vitro, cultured osteoarthritic
47 joint- origin canine synovial membrane cells are plastic adherant and fibroblast- like, and contain
48 populations of cells that can undergo chondrogenesis (Warnock et al., 2011, Warnock et al 2013),
49 suggesting the presence of mesenchymal stem cells. *In vivo* (Smith et al., 2012; Tienen et al.,
50 2006) synovium also has the ability to form fibrocartilage ECM. Conversely, synoviocytes in
51 osteoarthritic joints secrete a number of inflammatory mediators and destructive matrix
52 metalloproteinases (Benito et al., 2005; Fiorito et al., 2005; Sutton et al., 2007), which could
53 inhibit *in vitro* fibrochondrogenic potential. For example, canine osteoarthritic joint-origin
54 synoviocytes produce less total collagen than normal joint-origin synoviocytes in monolayer
55 culture (Warnock et al., 2011). This limitation may not be present with improved culture
56 conditions (Warnock et al. 2013). For instance, osteoarthritic joint-origin synovial fluid stem cells
57 require culture as a micro-mass to undergo efficient *in vitro* chondrogenesis, compared to cells
58 derived from healthy joint fluid (Krawetz et al., 2012).

59 Thus, the purpose of this study was to evaluate and compare the fibrochondrogenic
60 potential of synoviocytes from osteoarthritic and normal canine joints that were cultured as
61 tensioned bioscaffolds under conditions previously shown to increase meniscal-like ECM content
62 in canine osteoarthritic joint-origin synoviocytes (Warnock et al., 2013). We hypothesized that

63 with the use of this tensioned culture system, there would be no difference in cell viability and
64 fibrocartilage-like ECM formation between tensioned synoviocyte bioscaffolds from normal
65 joints (normal joint- origin tensioned synoviocyte bioscaffolds, “nTSB”) and osteoarthritic joints
66 (osteoarthritic joint- origin tensioned synoviocyte bioscaffolds, “oaTSB”).

67

68 2. Materials and Methods

69 2.1 Tissue Harvest

70 With informed owner consent, synovium was obtained from 12 dogs with naturally
71 occurring clinical osteoarthritis as per Institutional Animal Care and Use Committee approval.
72 Dogs were treated for degeneration of the cranial cruciate ligament and medial meniscal injury
73 via exploratory arthroscopy, partial meniscectomy if indicated, and tibial plateau leveling
74 osteotomy. Synovial villi were arthroscopically harvested during routine partial synovectomy
75 using a tissue shaver (Stryker, San Jose, CA) as previously described (Warnock et al., 2012).
76 Synovial villi from the osteoarthritic joints were immediately placed in a 50ml polypropylene
77 tube containing 40mL of Dulbeccos’ Modified Eagle’s Media (DMEM, Invitrogen)) with 10%
78 fetal bovine serum (FBS, Invitrogen), warmed to 37°C. The tube was transported immediately to
79 the laboratory and centrifuged at 313xg, media was decanted, and tissue fragments transferred by
80 pipette and sterile forceps into a digestion solution as described below.

81 Normal synovium was also harvested from six dogs which were euthanatized via sodium
82 pentobarbital overdose for reasons unrelated to the study, as per the Institutional Animal Care and
83 Use Committee Protocol and in accordance with the American Veterinary Medical Association
84 Humane Euthanasia Guidelines. Dogs were assessed by a Diplomate of the American College of
85 Veterinary Surgeons – Small Animal to not have any orthopedic disease based on medical history,

86 pre-mortem physical examination, and post- mortem gross joint evaluation. Post- mortem, a
87 lateral arthrotomy and patellar tendon transection was performed on each stifle joint. The
88 parapatellar, suprapatellar, lateral, and medial wall synovium were dissected off the joint capsule
89 using a #15 bard parker blade. Synovium was transported as described above. In the laboratory,
90 synovium harvested from normal joints was additionally was minced into 2x3mm pieces using
91 sterile technique.

92 2.2 Cell Culture

93 Osteoarthritic joint- origin synovial villi and normal joint- origin synovium tissue
94 fragments were completely digested with sterile Type 1A clostridial collagenase 10mg/mL in
95 RPMI 1640 solution (Invitrogen) over 2-6 hours at 37°C. Tissue was deemed to be completely
96 digested when no ECM could be visualized microscopically at 20x objective magnification. Cells
97 were cultured in monolayer for four passages to isolate Type B fibroblast-like synoviocytes
98 (Vasanjee et al., 2008) and Type C intermediate synoviocytes (Vasanjee et al., 2008) as described
99 previously (Warnock et al., 2012). The following media formulation was used for the duration of
100 culture: high glucose DMEM, supplemented with 17.7% FBS, 0.021 mg/mL glycine,
101 0.025mg/mL L-alanine, 0.037mg/mL L- asparagine, 0.038mg/mL L-aspartic acid, 0.042mg/mL
102 L-glutamic acid, 0.033 mg/mL L-proline, 0.030mg/mL L-serine, 0.23mg/mL pyruvate,
103 0.52mg/mL L-glutamine, 6.75mg/mL HEPES buffer, 177.0 units/mL penicillin, 177.0 µg/mL
104 streptomycin, and 0.44 ug/mL amphotericin). The flasks were incubated at 37°C, 5% CO₂, 95%
105 humidity, with sterile media change performed every 24 hours.

106 Cell flasks were observed under 10x objective magnification every 24 hours to assess
107 confluency. Cells were passaged upon reaching 95% confluence, which was defined as
108 monolayer cell culture with no visible exposed flask surface in between cells, and no overlap of
109 the cells on each other. At harvest and at each passage cell viability counts were performed using

the trypan blue exclusion assay (Strober, 2001). At the 4th passage, cells from each joint were transferred into eight 150cm² flasks and allowed to become hyperconfluent cell sheets, defined as cells overlapping each other in greater than 100% confluency. TSB were then made as previously described (Warnock et al., 2013). Briefly, hyperconfluent cell sheets were dislodged off the flask floors (Ando et al., 2008), and each sheet was wrapped over 2.0 cm diameter, 22ga cerclage wire hoops in three layers, with approximately 0.5 N of tension to avoid tearing, to synthesize TSB. The TSB were placed in 6-well plates in 9.0 mL of the above described culture media, with the free end of the cell sheet facing down to prevent loosening. Bioscaffolds were harvested for analysis after a total of 30 days in culture (Ando et al., 2008; Tan et al., 2010).

2.3 Bioscaffold Analyses

Bioscaffolds analyses examined presence of ECM components responsible for meniscal form and function. These include type I collagen, which, in the bovine, constitutes up to 98% of meniscal collagen, (Eyre and Wu, 1983) and subjectively, in the dog, accounts for the preponderance of meniscal collagen (Kambic and McDevitt, 2005); type II collagen, which accounts for a very small proportion of meniscal collagen (Eyre and Wu, 1983); α - smooth muscle actin (ASM) (Ahluwalia et al., 2001; Kambic et al., 2000; Spector, 2001); and glycosaminoglycans (GAG) (Adams and Ho, 1987; Nakano et al., 1997; Stephan et al., 1998), including aggrecan (Valiyaveetil et al., 2005). Differences in expression of inflammatory mediators or presence of macrophages were investigated as these factors may be associated with decreased in vitro ECM synthesis in osteoarthritic joint-origin synoviocytes (Fiorito et al., 2005; Pei et al., 2008a).

Cell Viability: One TSB per dog was washed three times in sterile phosphate buffered saline and immersed in 4 μ M ethidium homodimer and 6 μ M acetomethoxy calcein (calcein –AM)

133 solution (Ethidium homodimer and Calcein AM Live/Dead Viability Assay, Invitrogen, Carlsbad,
134 CA) for 20 minutes at 37°C, 5% CO₂, 95% humidity. Cells were then visualized in at least five
135 regions of the bioscaffolds, (and two in the center and three on the periphery, at approximately
136 the 2, 6, and 10 o'clock positions) using a laser microscope (Eclipse Ti-u Laser Microscope,
137 Nikon, Japan). The number of viable (green) and non- viable (red) cells per each field counted
138 by hand. Due to the complex three –dimensional nature of the bioscaffolds, these cell counts
139 provided an estimate of cell viability.

140 *Immunohistologic Analysis:* Two TSB per dog were fixed in 10% buffered formalin,
141 paraffin embedded, and tissue blocks cut in 4µm sections for histologic and immunohistologic
142 analysis. All slides were labelled with randomly generated acquisition numbers and analyzed in a
143 blinded fashion. Sections were stained with Hematoxylin and Eosin (“H&E”), Masson’s
144 Trichrome, and Toluidine Blue. Cell morphology and general ECM architecture was assessed
145 using H&E; organization and intensity of collagen staining was described using Masson’s
146 Trichrome, and intensity of GAG staining was assessed using Toluidine Blue.

147 *Immunohistochemistry:* Immunohistochemistry was performed as previously described
148 (Warnock et al., 2012) for type I collagen (AB749P; 1:100 dilution; Millipore), type II collagen
149 (AB746P; 1:100: Millipore), macrophage MAC387 receptor to determine type A synoviocyte
150 content, (CBL260; 1:200 dilution; Millipore); and alpha smooth muscle actin (M0851; 1:30;
151 Dako). Extracellular and intracellular immunoreactivity intensity and prevalence was scored as
152 previously described ([Wakshlag et al. 2011](http://dx.doi.org/10.7287/peerj.preprints.475v1)) with some modifications: immunoreactivity was
153 localized to intracellular or extracellular staining, and ECM immunoreactivity intensity was
154 described and scored, as negative (0), mild (1), moderate (2), or strong (3) staining. As
155 determined by hand count, intracellular immunoreactivity and extracellular immunoreactivity

156 was categorized as positive in <10%, 10-50%, or >50% of cells and sample area, respectively.
157 Each of these histologic observations was assigned a score (Table 2). Then a histologic intensity
158 coefficient was calculated for each ECM component, as follows: $[(\text{Extracellular matrix staining}$
159 $\text{intensity score}) \times (\text{percentage area coverage of positive staining score})] + [(\text{Intracellular staining}$
160 $\text{intensity score}) \times (\text{percentage positive staining cells score})]/2$ (Table 1).

161 *Tissue Weight:* One TSB per dog was lyophilized and a dry weight obtained. Samples were
162 digested in 1.0ml Papain Solution (2mM Dithiothreitol and 300ug/ml Papain) at 60°C in a water
163 bath for 24 hours. This papain digest solution was used to obtain double stranded DNA (dsDNA),
164 GAG and collagen content of the bioscaffolds.

165 *DNA Quantification:* Double stranded DNA quantification assay (The Quant-iT
166 PicoGreen™ Assay, Invitrogen) was performed per manufacturer's instructions; double stranded
167 DNA extracted from bovine thymus was used to create standards of 1,000, 100, 10, and 1 ng/mL.
168 Standard and sample fluorescence was read by a fluorometer (Qubit, Invitrogen) at 485nm
169 excitation/ 528nm emission, and dsDNA concentration was determined based on the standard
170 curve.

171 *Biochemical ECM Analysis:* Glycosaminoglycan content was determined by the di-methyl-
172 methylene blue sulfated glycosaminoglycan assay (Farndale et al., 1986) using a
173 spectrophotometer (Synergy HT– KC4 Spectrophotometric Plate Reader and FT4software,
174 BioTec, Winooski, VT). Collagen content was determined by Erlich's hydroxyproline assay, as
175 described by Reddy et al. (Reddy and Enwemeka, 1996). Hydroxyproline content was converted
176 to collagen content using the equation: $\mu\text{g hydroxyproline} \times \text{dilution factor} / 0.13 = \mu\text{g collagen}$
177 (Ignat'eva et al. 2007), because hydroxyproline consists of approximately 13% of the amino

acids in human meniscal collagen (Fithian, et al., 1990). Collagen and GAG content were standardized to tissue dry weight as percentage of dry weight, to compare the experimental neotissues to previously reported normal meniscal ECM content (Eyre and Wu, 1983). Total GAG and collagen content were also reported in $\mu\text{g}/\text{neotissue}$ to measure total synthetic activity over the course of 30 days in each TSB. GAG and collagen content were additionally standardized to dsDNA content using the following equations: $[\mu\text{g GAG}/\text{ug dsDNA}]$ (Li and Pei, 2011) and $[\mu\text{g collagen}/\text{ug dsDNA}]$ to identify chondrogenic cellular activity of each tested cell origin.

Real-Time RT-PCR: One TSB per dog was snap frozen in liquid nitrogen and stored at -80°C . Total RNA was isolated using the phenol-chloroform extraction (Chomczynski P, 1986) with slight modifications. Samples were pulverized using a liquid nitrogen-cooled custom-made stainless steel pulverizer and homogenized in trizol (Trizol, Qiagen Sciences, $0.025\text{mL}/\text{mg}$ of tissue) and mixed with chloroform. The aqueous phase was then treated with isopropanol to precipitate nucleic acids. RNA of samples was purified using on-column DNase digestion (RNeasy, Qiagen Sciences).

The RNA quality and quantity was determined using capillary electrophoresis (RNA 6000 Nano LabChip Kit, Agilent 2100 Bioanalyzer, Agilent Technologies), and RNA integrity numbers (Imbeaud S, 2005) were determined (2100 Expert software, Agilent Technologies).

First-strand cDNA synthesis was performed from 400 ng total RNA (SuperScript III First-Strand Synthesis System, Invitrogen Life Technologies, Carlsbad, CA) and Oligo-(dT)20 primers. To control for possible genetic DNA contamination, non reverse-transcribed samples were also processed.

Pre-designed primers and probes (Taq-Man® Primers and Probes, Applied Biosystems Inc., Foster City, CA) were obtained for each of the genes of interest: IL-1 β , IL-6, TNF- α , SOX-9 (an embryonic chondrogenic transcription factor), collagen type I α 1, collagen type II α 1, aggrecan, and the reference gene GAPDH (see Appendix 1). All assays were confirmed to amplify their targets at 95% or greater efficiency using RNA from tissues of interest. Quantitative real-time PCR was performed (StepOnePlus RT-PCR System, Applied Biosystems Inc.) using a proprietary reagent system (TaqMan Gene Expression Master Mix, Applied Biosystems Inc.) Controls included template-free negative controls and non reverse-transcribed negative controls. All samples were run in triplicates and all negative controls were run in duplicates for 40 cycles (15 seconds at 95°C, 1 minute at 60°C) after 2 minutes of incubation with Uracil-DNA Glycosylase at 50°C, and 10 minutes at 95°C of enzyme activation.

Quantitative gene expression was determined in triplicates using the comparative CT method (Schmittgen TD, 2008). The gene GAPDH was used as internal control (housekeeping gene). Threshold cycles (CT) for each gene were defined by recording the cycle number at which fluorescence reached a gene-specific threshold. Fold changes for gene expression data were calculated using the following formula: fold change = $2^{-\Delta\Delta CT} = [(C_{T\text{gene of interest}} - C_{T\text{housekeeping gene GAPDH}})_{\text{nTSB}} - (C_{T\text{gene of interest}} - C_{T\text{housekeeping gene GAPDH}})_{\text{oaTSB}}]$.

2.4 Statistical Methods

A D'Agostino & Pearson omnibus normality test was performed on all data to test for normality. Cell harvest data was non- parametric data and was analyzed with a Wilcoxon matched-pairs signed rank test, and data reported as median and interquartile range. Significance

221 was declared at $P < 0.05$. Data were analyzed with a statistical software program Graph Pad
222 Prism, San Diego, CA.

223 The effect of osteoarthritis (osteoarthritic versus normal joint status) on gene expression
224 and ECM composition was analyzed using a 2-tailed Student's *t*-test, assuming unequal
225 variances. The effect of osteoarthritis on the histologic scoring of TSB extracellular matrix
226 formation was analyzed using a non-parametric Mann-Whitney U-test; ranking of the histologic
227 scores was performed using a Kruskal-wallis analysis on ranks followed by a Fisher's exact test.
228 Significance was declared at $P < 0.05$. Data were analyzed using Statistical Analysis System,
229 version 9.3 (SAS Institute Inc., Cary, NC).

230

231 3. Results

232 3.1 Cell Harvest:

233 The mean age of dogs with stifle osteoarthritis was 4.7 years (range: 2-8 years). Breeds
234 represented included: Golden Retriever (1), American Staffordshire Terrier (2), Labrador
235 Retriever (3), Australian Shepherd (1), Rottweiler (2), Boston Bull Terrier (1), Goldendoodle (1),
236 and mixed breed (1), with 7 neutered males, 4 spayed females, and one intact female dog. As
237 observed by a Diplomate of the American College of Veterinary Surgeons – Small Animal, all
238 dogs had marked villous synovial hyperplasia and osteophytosis, and grade 1-2 Outerbridge
239 cartilage lesions of the medial femoral condyle and tibial plateau (Outerbridge, 1961). Cell yield
240 from arthroscopic synovial debris was $1.9 \times 10^6 \pm 3.7 \times 10^5$ cells per joint, and cells were 99.5%
241 ± 0.002 viable at harvest.

Mean age of dogs with normal stifles was 4.3 years (range: 3-6 years); breeds represented included: Red Tick Hounds (4), Labrador Retriever (1), and American Staffordshire cross (1), with 3 female intact dogs, 2 male intact dogs, and one neutered male. Cell yield per joint was $1.4 \times 10^7 \pm 2.6 \times 10^6$ per joint and cells were $99.5\% \pm 0.01$ viable. As the entire stifle joint synovial membrane could be harvested post mortem, a greater volume of tissue and thus greater cell numbers were obtained from the normal joints versus arthroscopic harvest of the osteoarthritic joints ($P=0.01$).

3.2 Cell Culture and Cell Characterization:

At 4th passage, cells were transferred into eight 150cm² flasks in order to have enough TSB for tissue analyses. This, however, resulted in greater cell seeding numbers for nTSB versus oa TSB. Thus, normal joint-origin synoviocytes were seeded at 1.49×10^7 cells per flask, whereas 6.52×10^6 osteoarthritic joint-origin cells were seeded per flask. At 4th passage, normal joint-origin cells were $99.0 \pm 0.4\%$ viable compared with $98.8 \pm 0.4\%$ viability of osteoarthritic joint-origin cells ($P=0.85$). Culture duration from tissue harvest to hyperconfluent cell membrane formation and synthesis of TSB was 37.6 days and similar for both cell origins (range 20-49 days).

During the first week of tensioned bioscaffold culture, the culture media phenol red pH indicator changed to yellow by the time the 24 hour media change was required, indicating marked increase in media acidity. In addition, during the first 7-10 days of culture, approximately 2-3 bioscaffolds per normal and osteoarthritic joint unraveled or slipped off their wire hoops (no group differences observed), and were not analyzed in this study. The typical appearance of intact nTSB and oaTSB is pictured in Fig. 2; thickness of TSB was 2-3mm.

265 At harvest, tensioned bioscaffolds from normal dogs had a dry weight of 39.3mg (range
266 27.5-50.4mg), which was more than for oaTSB (23.6mg, range 10.2-50.1mg; $P = 0.008$).

267 Mean estimated cell viability of nTSB and oaTSB was similar, with 78% of cells viable
268 (range: 72-86%). Cell viability was not associated with peripheral versus central location on the
269 TSB. Laser microscopy revealed cells with fusiform, fibroblastic cytoplasm, oriented parallel
270 with the vector of tension, as well as the presence of acellular, circular regions in the bioscaffolds.
271 Hematoxylin and eosin staining revealed highly cellular bioscaffolds, with layers of fibroblastic
272 cells organized in parallel, as sheets or bands, or variably arranged in whorls, with eosinophilic
273 ECM (Fig.3). Subjectively, both nTSB and oaTSB had heterogeneous extracellular matrix
274 architecture and cell distribution, with regions of dense cellularity and regions of dense
275 extracellular matrix (Fig. 3).

276 Percent dsDNA content was used to quantify tissue cellularity. Despite an initial higher
277 seeding cell count at 4th passage, dsDNA accounted for $0.11 \pm .02\%$ of nTSB dry weight, versus
278 $0.21 \pm 0.03\%$ of oaTSB dry weight ($P = 0.01$).

279 Immunohistologically, oaTSB had more ASM positive cells than nTSB; the median
280 histologic score for nTSB was 6 versus 9 for oaTSB ($p = 0.0102$, Fig.4). Nine of 12 oaTSB had
281 the highest possible ASM histologic scores of 9, whereas none of the nTSB achieved a perfect
282 score of 9 ($P = 0.009$, Fig.4). In 50% of all bioscaffolds, ASM positive cells were concentrated
283 around the bioscaffold periphery and around the margins of what appeared to be spontaneously
284 forming circular defects ranging from 70-600 μ m (Fig.3). These circular defects corresponded
285 with the acellular regions viewed on laser microscopy. The other 50% of bioscaffolds did not
286 contain circular defects, nor did ASM expression seem to be geographically localizable.

Gene Expression: The oaTSB had a greater gene expression of type I collagen (7-fold increase; $P = 0.04$) and type II collagen (71-fold increase; $P = 0.02$) and a lower gene expression of interleukin-6 (19-fold decrease; $P = 0.001$) versus nTSB. No significant changes were observed for relative expression of SOX-9 ($P=0.72$), aggrecan ($P=0.84$), and tumor necrosis factor- α ($P=0.77$; Table 2). Interleukin-1 β was not expressed at detectable levels in any bioscaffolds.

Glycosaminoglycan Content: The total GAG content of oaTSB was lower than the GAG content of nTSB ($P=0.02$; Table 3). After adjustment for dry weight or DNA content, no significant group differences were observed.

Glycosaminoglycan was deposited regionally in all bioscaffolds but more GAG staining was observed in oaTSB than in nTSB. Median GAG histologic score was 1.0 for nTSB and 3.0 for oaTSB ($P=0.0007$, Figs. 4,5). Only 1 of 6 nTSB had a GAG histologic score above 1, whereas 11 of 12 oaTSB had GAG histologic score above 1 ($P=0.004$, Fig.4).

Collagen Content: There was no difference in quantified total collagen content of oaTSB and nTSB (Table 3). Similar results were observed after adjustment for dry weight or DNA content.

Masson's Trichrome staining revealed collagen deposited in bands, sheets, and whorls, containing and surrounded by numerous fibroblastic cells lined in parallel with the orientation of the collagen (Fig.6). A significant difference in the median type I collagen histologic scores of nTSB and oaTSB could not be detected, which were 7.5 and 6.0, respectively ($P=0.11$, Fig.4).

307 However, 4 of 6 nTSB had a type I collagen histologic score greater than 7.5, versus only 1 of 12
308 oaTSB had a collagen score of 7.5 ($P=0.02$, Fig.4). Histologically, nTSB had more type II
309 collagen than oaTSB (Fig.6); median type II collagen histologic scores were 4.0 in nTSB and 2.5
310 in oaTSB, ($P= 0.03$, Fig. 4). None of the oaTSB had a score greater than 2.5 whereas 5 of 6 nTSB
311 had a collagen type II histology score of 2.75 ($P=0.0007$, Fig 4).

312 *Synovial Macrophage Content:* Based on immunohistochemistry, no macrophages
313 (Type A synoviocytes) were found in any bisocaffolds (Fig.7).

314 4.0 Discussion

315 Previous studies comparing *in vitro* canine synoviocyte fibrochondrogenesis in monolayer
316 culture (Warnock et al., 2011), and canine synoviocyte chondrogenesis in micromass culture
317 (Krawetz et al., 2012) concluded that osteoarthritic synoviocytes had inferior *in vitro*
318 fibrochondrogenic potential, compared with normal synoviocytes. Fiorito and workers came to a
319 similar conclusion in a study comparing *in vitro* chondrogenesis of human synoviocytes grown in
320 pellet culture, as determined by histologic analysis (Fiorito et al., 2005). In contrast, with the
321 culture conditions in the present study, especially providing conditions for self-tensioning, cells
322 originating from osteoarthritic joints increased type I and II collagen gene expression, and oaTSB
323 contained similar total collagen content, as compared to nTSB. While tissue dry weight and thus
324 total GAG content of oaTSB was lower than nTSB, a significant difference in GAG content
325 standardized to dry weight and cellularity could not be detected between oaTSB and nTSB.
326 Histologic analysis using toluidine blue, a semi-quantitative measure of GAG, revealed more
327 GAG deposition in oaTSB than nTSB. Thus, the greater dry weight of nTSB versus oaTSB was
328 likely due to unmeasured ECM components such as fibronectin, type III and VI collagen, and
329 vitronectin (Ando et al., 2007; Ando et al., 2008), which are found in native synovium (Okada et

330 al., 1990; Price et al., 1996). These findings also indicate that given the chance to self- tension,
331 autologous, diseased synoviocytes can produce the ECM components of fibrocartilage *in vitro* at
332 a comparable level of normal joint-origin synoviocytes.

333 The unstable mechanical environment and inflammatory environment of the cranial
334 cruciate ligament deficient joint favors synovial intimal hyperplasia and synovial membrane and
335 joint capsule fibrosis (Bleedorn et al., 2011; Buckwalter, 2000; Oehler et al., 2002; Smith et al.,
336 1997), all of which were encountered in the osteoarthritic joints in the present study. The *in vivo*
337 pathogenic synovial hyperplasia may have accounted for the collagen gene upregulation seen in
338 oaTSB. Rat and human osteoarthritic synoviocytes spontaneously express TGF β -1 and its
339 receptor (Fiorito et al., 2005; Mussener et al., 1997), which is a pro-collagen and chondrogenic
340 growth factor (Daireaux et al., 1990; Leask and Abraham, 2004; Miyamoto et al., 2007; Pangborn
341 and Athanasiou, 2005a, b; Pei et al., 2008b). Upregulation of TGF β -1 and its receptor may also
342 be a plausible mechanism for oaTSB collagen gene upregulation. Collagen II upregulation
343 seemed to occur independently of SOX-9 expression, a finding duplicated in cultured human
344 osteoarthritic chondrocytes (Aigner et al., 2003). Additionally, decreased expression of IL-6 gene
345 may be a mechanism for the observed upregulation of type II collagen genes in oaTSB; IL-6 has
346 been found to inhibit chondrogenic differentiation of murine marrow mesenchymal cells (Wei et
347 al., 2013). Further research is required to confirm the mechanism of hyaline chondrogenic ECM
348 formation in canine TSB, through immunohistochemistry of TGF β -receptor and SMAD-
349 family protein expression (Xu et al., 2012).

350 Despite equal quantities of non-specific collagen in nTSB and oaTSB, immunohistologic
351 analysis revealed less type I and type II collagen in oaTSB, particularly in the ECM. Post
352 translation regulation by prolyl-4-hydroxylases (Grimmer et al., 2006) or ECM degradation by

353 synovial matrix metalloproteinases (Fiorito et al., 2005) may have decreased oaTSB
354 accumulation of type I and II collagen accumulation, despite increased collagen gene expression.
355 IL-6 has also been found to increase gingival fibroblast synthesis of type I collagen in vitro
356 (Martelli-Junior et al., 2003), and increase type I collagen synthesis by tenocytes in vivo
357 (Andersen et al., 2011). It is possible that the decreased IL-6 gene expression in oaTSB
358 synoviocytes also decreased type I collagen formation as seen on histologic analysis. One
359 weakness of our study was that expression of type I and II collagen was not corroborated with a
360 Western blot, nor quantified via ELISA, to further our understanding of this discrepancy between
361 histologic collagen expression and collagen gene expression. Additionally we did not characterize
362 the percentage and type of mesenchymal progenitor cells present in normal versus osteoarthritic
363 synovium; difference in number and chondrogenic potential of these cells may have also
364 accounted for a difference in collagen ECM formation.

365 Other osteoarthritic cell types, such as chondrocytes, have reduced cell proliferation
366 compared to normal cells in monolayer culture (Acosta et al., 2006). In contrast, oaTSB
367 contained more dsDNA per dry weight than nTSB, despite the lower harvest cell yield and lower
368 cell seeding density at 4th passage of osteoarthritic joint- origin synoviocytes. There was an
369 intrinsic weakness of our study; by clinical necessity, synovium from osteoarthritic joints was
370 harvested using a different technique (arthroscopy) than the normal joints (arthrotomy), and more
371 synoviocytes can be obtained via arthrotomy. Although cell growth kinetics was not the focus of
372 this study, cell culture media containing 17.7% FBS likely provided mitotic stimuli to support
373 and increase oaTSB cellular proliferation. The markedly hyperplastic state of the synovium in
374 vivo may also have primed the osteoarthritic cells to continue to proliferate in vitro. Cell viability
375 was high at harvest and at the start of 4th passage, but declined in all TSB; due to the long culture
376 period, cell mortality may have been caused by senescence. Additionally, as evidenced by media

color changes, inadequate nutrient delivery to TSB in the culture wells and daily shifts in pH may have also led to nTSB and oaTSB cell mortality. This cell mortality may have affected ECM formation in both groups: the collagen content of nTSB (12%) and oaTSB (16%) did not reach that of the healthy meniscus, at 60-70% of dry weight (McDevitt and Webber, 1990), although the GAG content of nTSB (1.7%) and oaTSB (2%) did approximate the 2-3% GAG per dry weight of the whole meniscus (McDevitt and Webber, 1990; Stephan et al., 1998).

Consistent with prior studies (Warnock et al. 2013), all oaTSB and nTSB in the present study were negative for any macrophages, which have been reported to contaminate human osteoarthritic synoviocyte monolayer cultures and reduce *in vitro* chondrogenic activity (Pei et al., 2008a) by contributing to the inflammatory milieu. In the present study, 4 passages and long term culture as TSB likely eliminated any non-adherent cells, including synovial macrophages (Krey et al., 1976). Synovium from osteoarthritic joints has also been found to express inflammatory cytokines (Fiorito et al., 2005). Both nTSB and oaTSB expressed similar RNA quantity of the TNF α gene, indicating an inflammatory response in *in vitro* culture (Lindroos et al., 2010), independent of the diseased status of the cell origin. Paradoxically, IL-6 expression was decreased in oaTSB. Although the exact reason for this is unclear, decreased IL-6 gene expression may represent the response of synoviocytes from osteoarthritic joints to the change in environment; from the high motion, inflamed stifle containing multiple injured cell types (ligament, cartilage, meniscus, synovium) to the static tension of TSB culture and high FBS concentration cell culture media.

Decreased IL-6 in oaTSB may have reflected better mechanical homeostasis (Asparuhova et al., 2009; Chan et al., 2011; Gardner et al., 2012) in the cells in oaTSB: the majority of cells in oaTSB were uniformly positive for ASM, while 10-50% of nTSB cells were ASM positive. Synoviocytes increase expression of intracellular ASM in response to TGF β -1 (Xu et al., 2012).

401 Endogenous receptivity in osteoarthritic origin-joint synoviocytes to TGF β -1 present in FBS
402 (Goddard et al., 1990; Mussener et al., 1997) may explain increased ASM in the cells of oaTSB.
403 In the present study, staining for ASM was positively associated with the formation of circular
404 defects, indicating that the ECM was not strong enough to prevent tears from forming during
405 ASM-mediated self-tensioning (Kambic et al., 2000; Vickers et al., 2004; Warnock et al., 2013).
406 Given the higher dsDNA content of oaTSB and the high cellularity of the TSB, these defects may
407 have also been caused by increased cell turnover.

408 Conclusion: When cultured as TSB in high concentrations of FBS, osteoarthritic joint-
409 origin synoviocytes can produce ECM components of meniscal fibrocartilage at similar levels to
410 normal joint- origin synoviocytes. Potential reasons for this include increased collagen and
411 decreased IL-6 gene expression and the greater GAG and ASM staining in oaTSB compared with
412 nTSB Osteoarthritic joint-origin synoviocytes are a viable cell source toward meniscal tissue
413 engineering. Further investigation of culture environments to optimize cell viability and ECM
414 formation and strength are justified due to the promising data reported here.

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Figure 1

Hyperconfluent cell sheets.

Representative example of a hyperconfluent cell sheet just prior to harvest for formation of a tensioned synoviocyte bioscaffold. A) gross appearance of the hyperconfluent cell sheet in monolayer culture, and B) phase contrast photomicrograph of the hyperconfluent cell sheet, 10X objective magnification, bar= 100 μ m.

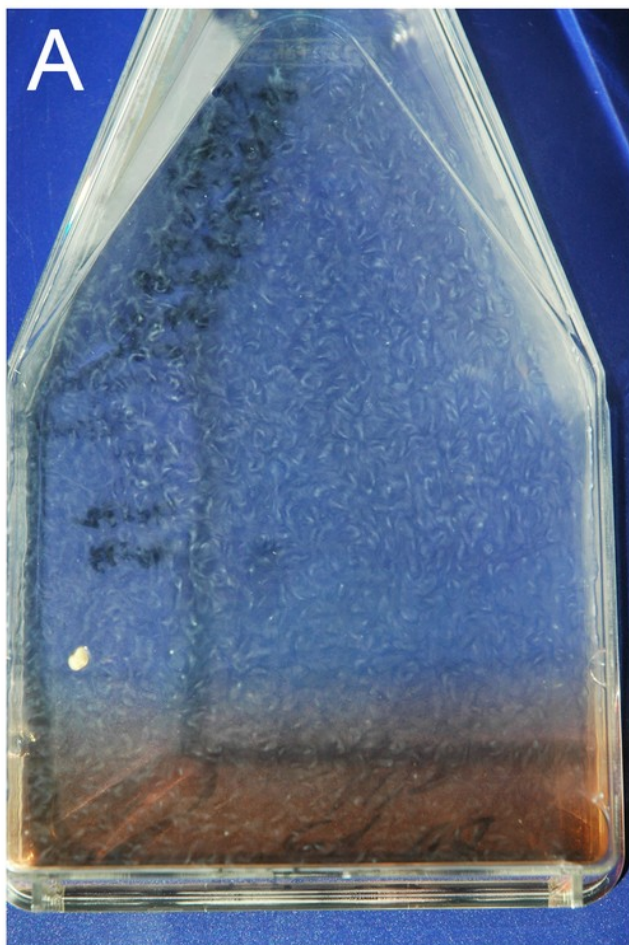


Figure 2

Tensioned synoviocyte bioscaffolds.

Representative samples of a tensioned synoviocyte bioscaffold synthesized from normal joint origin synoviocytes, or nTSB ("N"), and a tensioned synoviocyte bioscaffold from osteoarthritic joint origin

synoviocytes, or "oaTSB," ("OA").

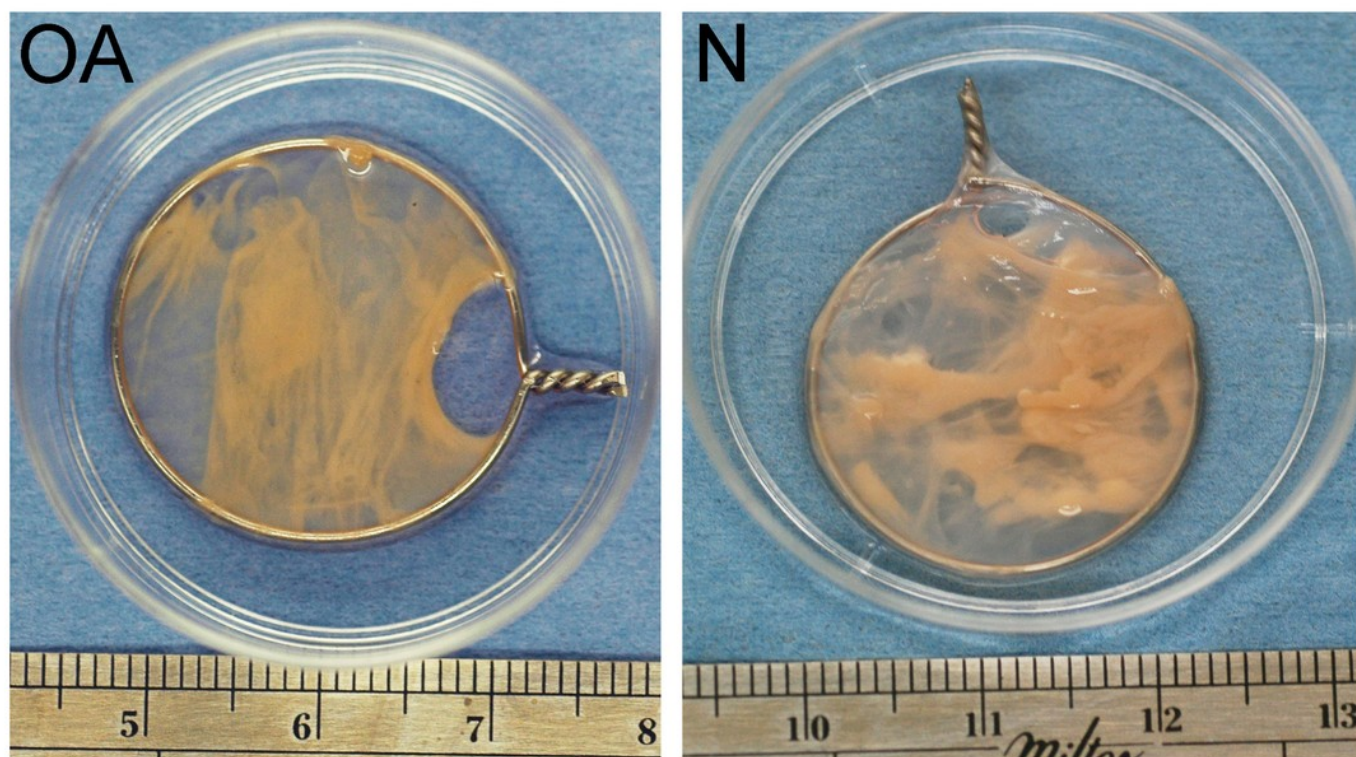


Figure 3

Bioscaffold cellularity and alpha- smooth muscle actin.

Hematoxylin and Eosin stain of normal joint-origin synoviocyte bioscaffolds ("N H&E ") and osteoarthritic joint-origin synoviocyte bioscaffolds ("OA H&E "). Immunohistochemistry for alpha smooth muscle actin (ASM) of normal joint-origin synoviocyte bioscaffolds ("N ASM ") and osteoarthritic joint-origin synoviocyte bioscaffolds ("OA ASM "). Note the more extensive expression of ASM in the osteoarthritic joint-origin synoviocyte bioscaffold cells, and regional strong ASM expression along the periphery of spontaneously forming defects (*) in the normal and osteoarthritic joint-origin bioscaffolds. Immunohistochemistry negative controls of normal joint-origin synoviocyte bioscaffolds ("N NC ") and osteoarthritic joint-origin synoviocyte bioscaffolds ("OA NC "). 10X objective magnification, bar= 100µm.

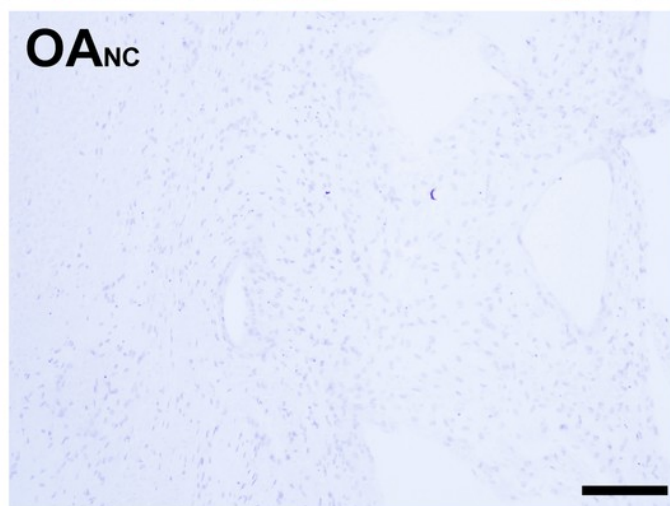
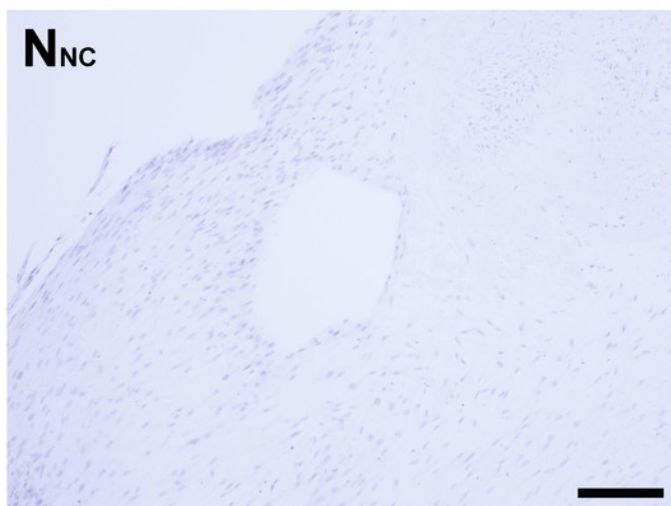
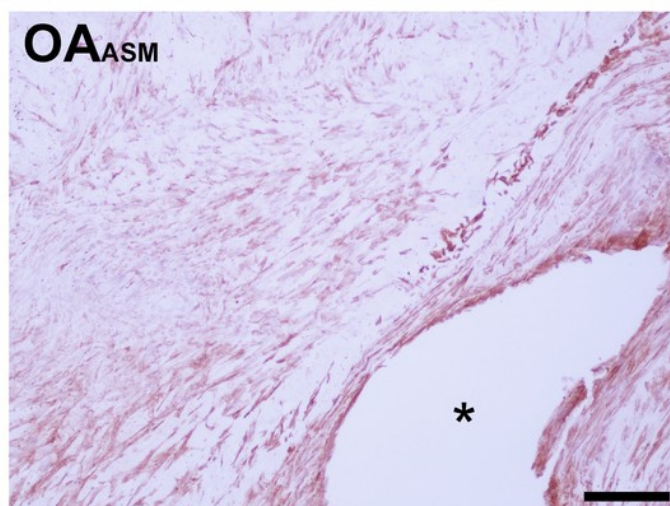
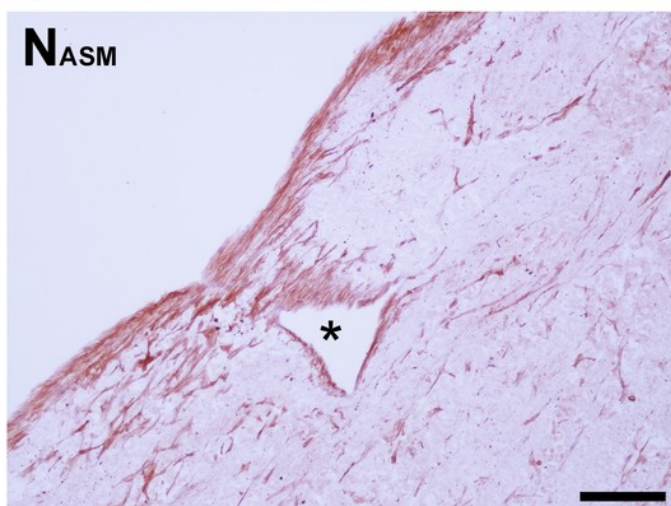
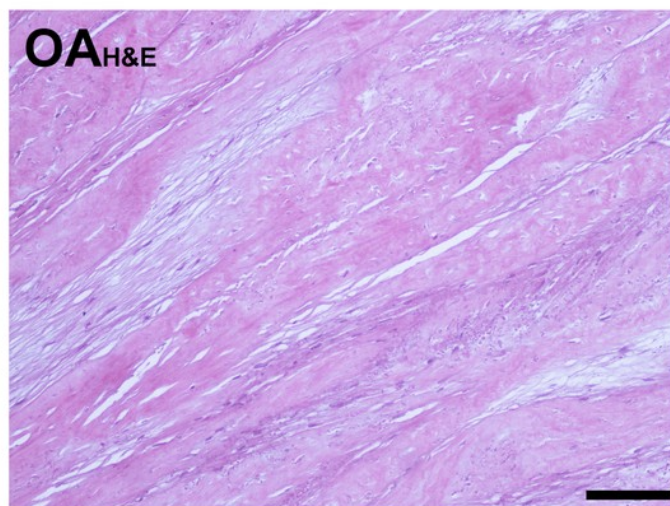
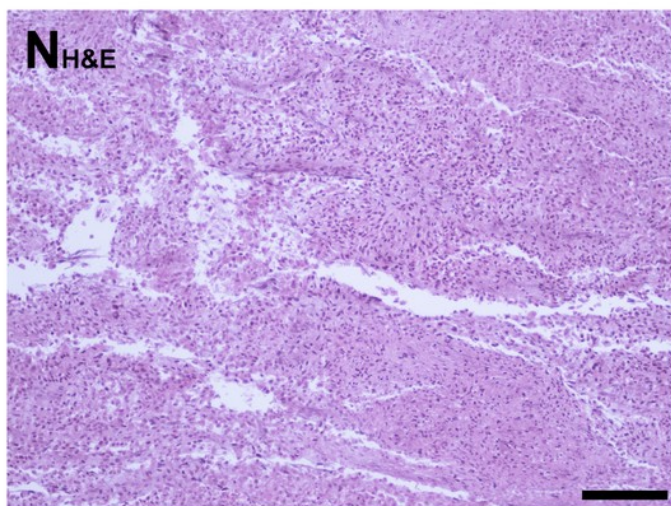


Figure 4

Histology scores for tensioned synoviocyte bioscaffolds

Histology scores for type I collagen, type II collagen, glycosaminoglycan, and alpha-smooth muscle actin in normal joint-origin tensioned synoviocyte bioscaffolds versus osteoarthritic joint-origin tensioned synoviocyte bioscaffolds, showing the median and interquartile range.

Histologic scores for collagens type 1 and 2 were calculated as follows: Histologic score = $\frac{[(\% \text{ positive staining cells} \times \text{intracellular staining intensity}) + (\% \text{ positive stained extracellular area} \times \text{extracellular staining intensity})]}{2}$. The histologic score for alpha smooth muscle actin (ASMA) was calculated by $(\% \text{ positive staining cells} \times \text{intracellular staining intensity})$. The histologic score for glycosaminoglycan (GAG) was calculated by $(\% \text{ positive stained extracellular area} \times \text{extracellular staining intensity})$. An (*) denotes statistical significance. [p]

Bioscaffold Histologic Scores

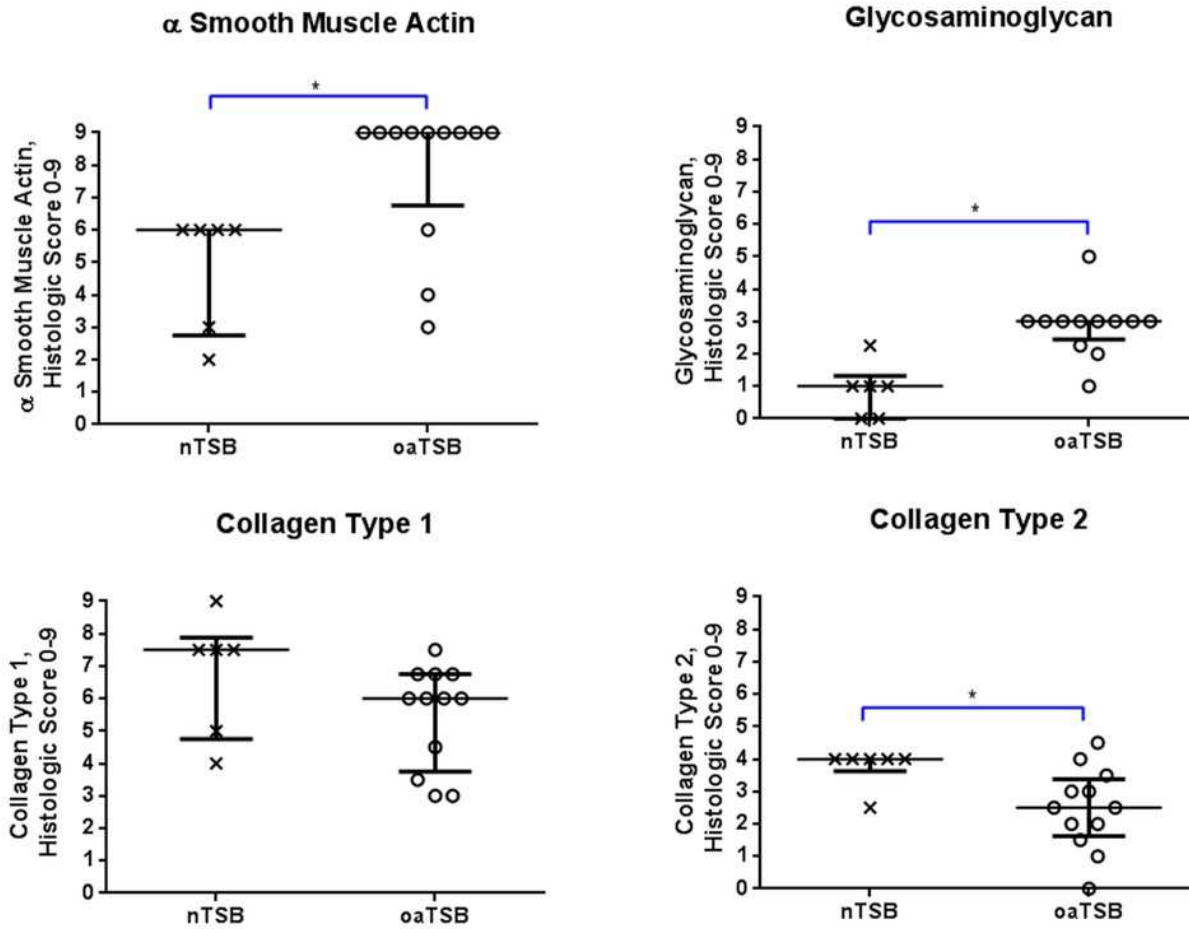


Figure 5

Histologic analysis of glycosaminoglycan content of tensioned synoviocyte bioscaffolds.

Histologic analysis of glycosaminoglycan content of tensioned synoviocyte bioscaffolds

Toluidine Blue staining for glycosaminoglycan of normaljoint-origin tensioned synoviocyte bioscaffolds (“NTB”) and osteoarthriticjoint- origin tensioned synoviocyte bioscaffolds (“OATB”). 10Xobjective magnification, bar= 100μm.

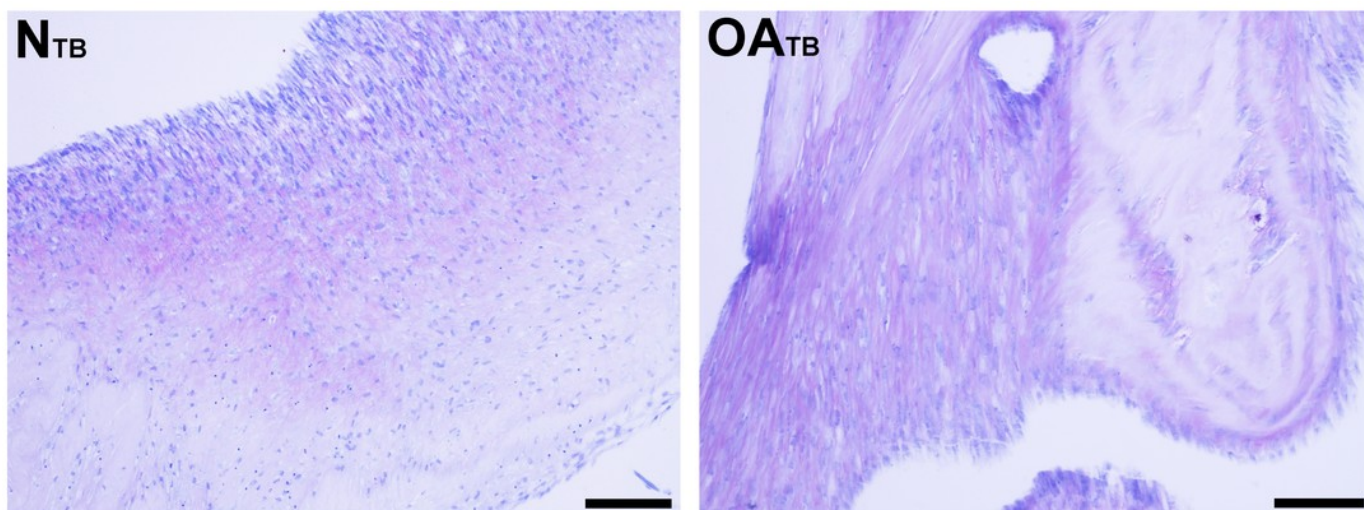


Figure 6

Histologic analysis of collagen content of tensioned synoviocyte bioscaffolds.

Masson's Trichrome staining for collagen of normal joint-origin synoviocyte bioscaffolds ("NMT") and osteoarthritic joint-origin synoviocyte bioscaffolds ("OA MT").

Immunohistochemistry for type I collagen and type II collagen of normal joint-origin synoviocyte bioscaffolds ("NCOL1" and "NCOL2") and osteoarthritic joint-origin synoviocyte bioscaffolds ("OACOL1" and "OACOL2"). In this example the type I collagen ECM of both bioscaffolds is moderately positive. For type II collagen, the cells are moderately immunoreactive and the ECM is mildly immunoreactive in the normal joint-origin synoviocyte bioscaffold, while the cells and ECM of the osteoarthritic joint-origin bioscaffold are mildly immunoreactive. 10X objective.

magnification, bar= 100µm.

[p]

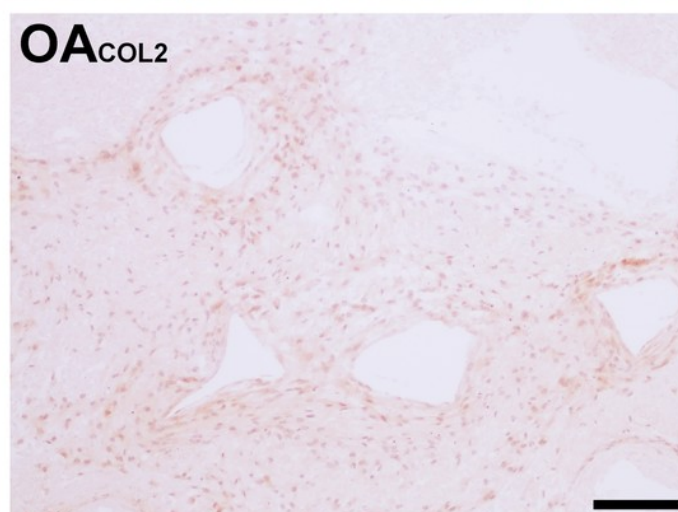
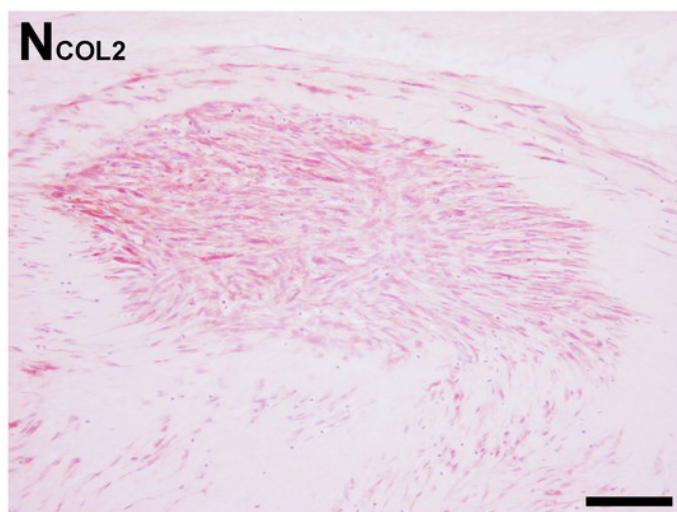
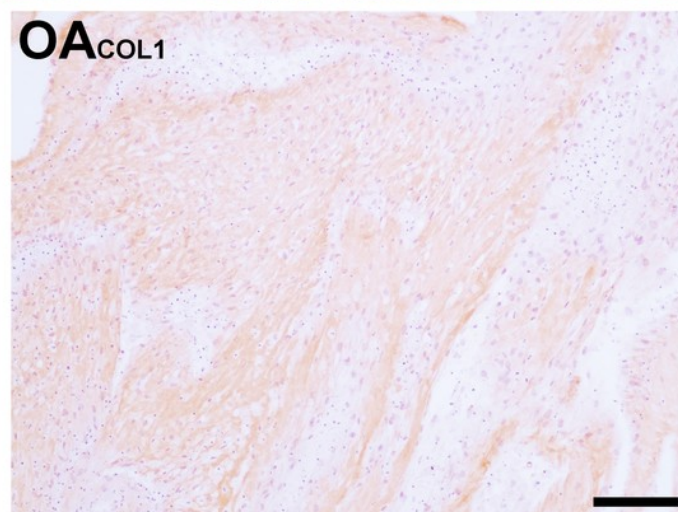
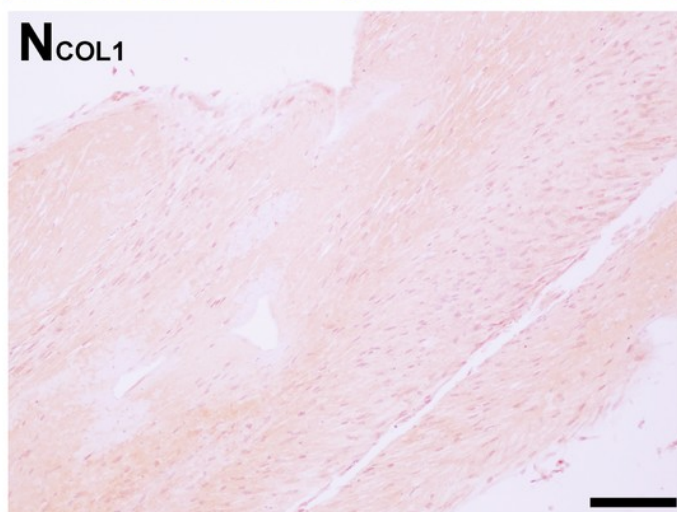
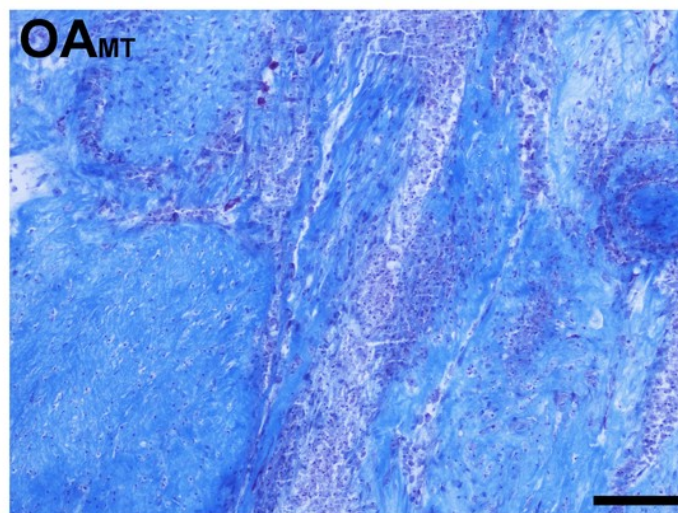
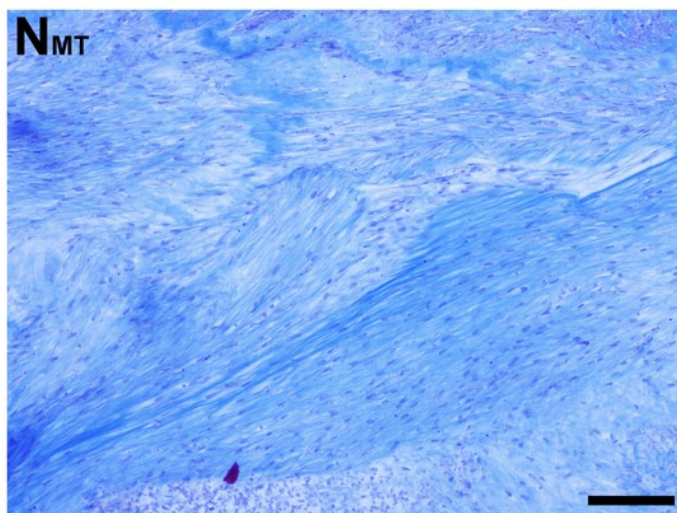


Figure 7

Immunohistochemical analysis for macrophages

Immunohistochemistry for the macrophage MAC387 receptor, to determine type A synoviocyte content, in a lymph node (positive control, "PC_{MAC}"), normal joint- origin bioscaffold ("N_{MAC}") and osteoarthritic joint-origin synoviocyte bioscaffolds ("OA_{MAC}").

Negative controls are as pictured in Fig.3. Note the lack of immunoreactivity in the bioscaffold samples. The positive control, a canine lymph node, is shown at 20X objective magnification, bar= 100µm, to allow clearer viewing of the macrophages. To show adequate representation of the bioscaffold neotissues, N_{MAC} and OA_{MAC} are pictured at 10X objective magnification, bar= 100µm.

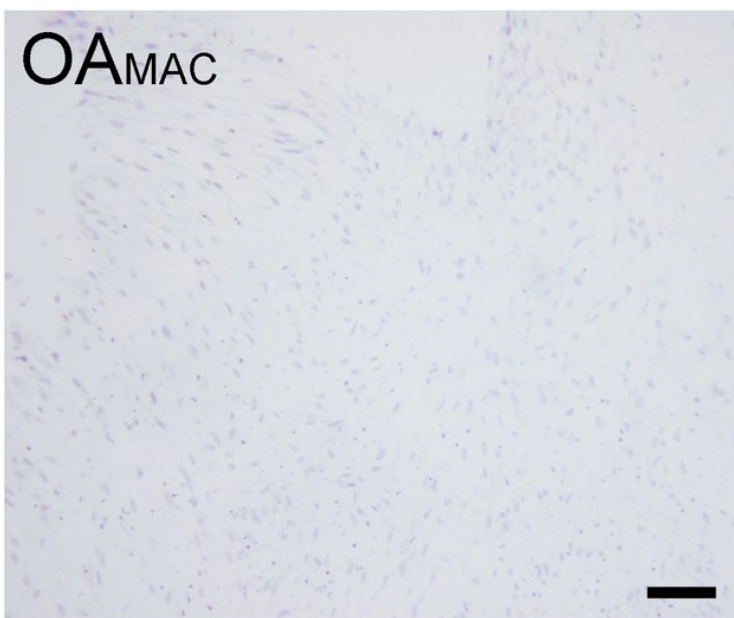
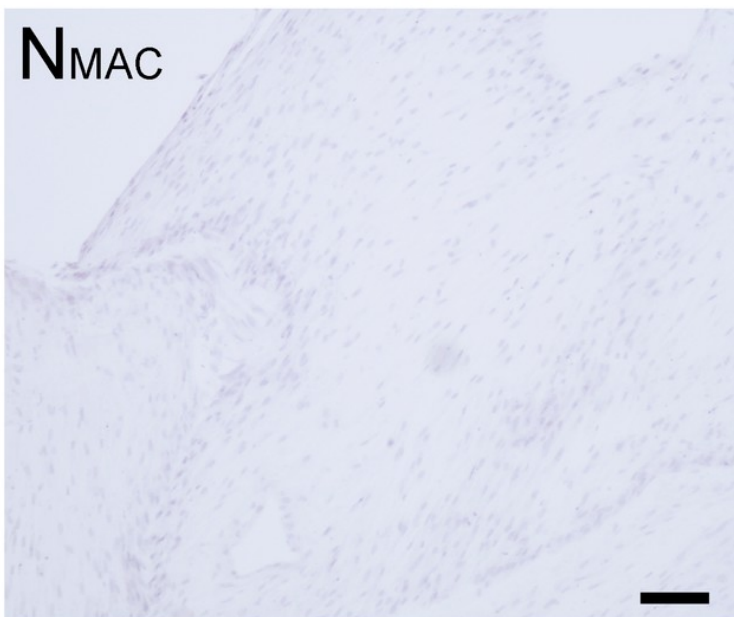
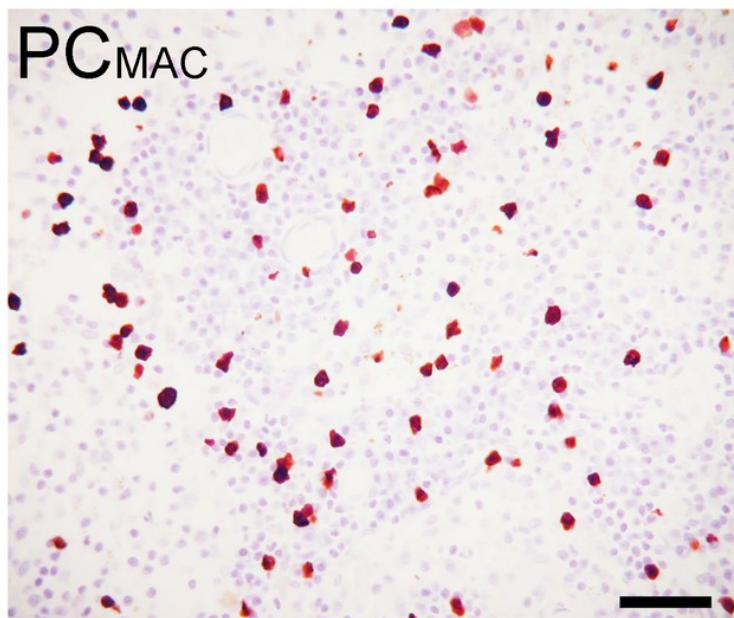


Table 1 (on next page)

Histologic scoring system

Extracellular and intracellular immunoreactivity intensity was localized to intracellular or extracellular staining, and ECM immunoreactivity intensity was described and scored as negative (0), mild (1), moderate (2), or strong (3) staining. As determined by hand count, intracellular immunoreactivity and extracellular immunoreactivity was categorized as positive in <10%, 10-50%, or >50% of cells and sample area, respectively. Each of these histologic observations was assigned a score (Table 2). Then a histologic intensity coefficient was calculated for each ECM component, as follows:
$$\frac{[(\text{Extracellular matrix staining intensity score}) \times (\text{percentage area coverage of positive staining score})] + [(\text{Intracellular staining intensity score}) \times (\text{percentage positive staining cells score})]}{2}$$
 (Table 1).

Table 1.

Histologic scoring system:

	% Positive staining cells				Intracellular staining intensity			
	None	<10%	10-50%	>50%	None	Mild	Moderate	Strong
Intracellular score	0	1	2	3	0	1	2	3
	% Positive stained extracellular area				Extracellular staining intensity			
	None	<10%	10-50%	>50%	None	Mild	Moderate	Strong
Extracellular score	0	1	2	3	0	1	2	3

Table 2_(on next page)

Gene expression in tensioned synoviocyte bioscaffolds

The effect of osteoarthritis on fibrochondrogenic gene expression of tensioned synoviocyte bioscaffolds (fold-changes $\pm$ SEM). Fold changes were calculated using the following formula:
fold change = $2^{-\Delta\Delta CT} = [(C_T \text{ gene of interest} - C_T \text{ housekeeping gene GAPDH})_{\text{oaTSB}} - (C_T \text{ gene of interest} - C_T \text{ housekeeping gene GAPDH})_{\text{nTSB}}]$.

Table 2: The effect of osteoarthritis on fibrochondrogenic gene expression of tensioned synoviocyte bioscaffolds (fold-changes $\pm$ SEM). Fold changes were calculated using the following formula: fold change = $2^{-\Delta\Delta CT} = [(C_T \text{ gene of interest} - C_T \text{ housekeeping gene GAPDH})_{\text{oaTSB}} - (C_T \text{ gene of interest} - C_T \text{ housekeeping gene GAPDH})_{\text{nTSB}}]$.

Dog	Tensioned Synoviocyte Bioscaffolds (TSB)		SEM	P-value
	Normal	Osteoarthritis		
Gene:	N = 4	N = 7		
SOX-9	0 ^{Reference}	+1.17	1.54	0.72
Collagen type I α 1	0	+6.88	2.62	0.04
Collagen type II α 1	0	+71.1	4.48	0.02
Aggrecan	0	-1.15	1.77	0.84
Interleukin-6	0	-19.0	2.01	0.001
Tumor Necrosis Factor α	0	+1.49	2.55	0.77

Table 3(on next page)

Extracellular matrix and dsDNA content of tensioned synoviocyte bioscaffolds

The effect of osteoarthritis on extracellular matrix and double stranded- DNA composition of tensioned synoviocyte bioscaffolds. Data is reported as mean $\pm$ SEM.

Table 3: The effect of osteoarthritis on extracellular matrix composition of tensioned synoviocyte bioscaffolds. Data is reported as mean $\pm$ SEM.

Dog:	Tensioned Synoviocyte Bioscaffolds (TSB)		P-Value
	Normal N = 6	Osteoarthritis N = 12	
Concentrations ($\mu\text{g}/\text{neotissue}$):			
Glycosaminoglycan	684 $\pm$ 74	434 $\pm$ 44	0.02
Collagen	4855 $\pm$ 1270	3302 $\pm$ 392	0.29
DNA	47.4 $\pm$ 11.9	42.3 $\pm$ 5.2	0.71
Proportion (% dry weight):			
Glycosaminoglycan	1.73 $\pm$ 0.11	2.05 $\pm$ 0.16	0.11
Collagen	12.1 $\pm$ 2.4	16.6 $\pm$ 2.4	0.20
DNA	0.111 $\pm$ 0.020	0.214 $\pm$ 0.030	0.01
Index ($\mu\text{g}/\text{ug dsDNA}$):			
GAG	18.7 $\pm$ 3.9	11.4 $\pm$ 1.6	0.13
Collagen	132 $\pm$ 36	92.0 $\pm$ 17.5	0.35