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Tissue Engineering of Ligaments: A Comparison of Bone Marrow Stromal Cells, Anterior Cruciate Ligament, and Skin Fibroblasts as Cell Source*

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ABSTRACT

Anterior cruciate ligament (ACL) reconstruction surgery still has important problems to overcome, such as “donor site morbidity” and the limited choice of grafts in revision surgery. Tissue engineering of ligaments may provide a solution for these problems. Little is known about the optimal cell source for tissue engineering of ligaments. The aim of this study is to determine the optimal cell source for tissue engineering of the anterior cruciate ligament. Bone marrow stromal cells (BMSCs), ACL, and skin fibroblasts were seeded onto a resorbable suture material [poly(L-lactide/glycolide) multifilaments] at five different seeding densities, and cultured for up to 12 days. All cell types tested attached to the suture material, proliferated, and synthesized extracellular matrix rich in collagen type I. On day 12 the scaffolds seeded with BMSCs showed the highest DNA content ($p < 0.01$) and the highest collagen production ($p < 0.05$ for the two highest seeding densities). Scaffolds seeded with ACL fibroblasts showed the lowest DNA content and collagen production. Accordingly, BMSCs appear to be the most suitable cells for further study and development of tissue-engineered ligament.

INTRODUCTION

RUPTURE OF THE ANTERIOR CRUCIATE LIGAMENT (ACL) is one of the most common sports-related injuries, with approximately 95,000 cases per year in the United States.¹ It is estimated that more than 50,000 ACL reconstructions are performed each year in the United States,¹ making it one of the most commonly performed surgical procedures in sports medicine. Because of the poor long-term results of synthetic grafts,^{2,3} biologic grafts are most frequently used for ACL reconstructions.

However, biological grafts still have considerable drawbacks, such as donor site morbidity (autografts) and the risk of disease transmission (allografts).^{4,5} Tissue engineering could potentially provide a solution for the limitations described. The basic principle of this technology has been firmly established^{6,7} and its application would dramatically improve clinical practice in ligament reconstructive surgery.

The aim in tissue engineering of ligaments is to provide an implant that results in a fully regenerated living autologous ligament with long-term viability. Our goal is

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to reconstruct a ligament by providing a resorbable scaffold seeded with cells, that induces neoligament formation that adequately meets the required biological and mechanical properties.

To date, as compared with bone or cartilage tissue engineering, limited information is available on tissue engineering of tendons or ligaments.^{8–16} Various biodegradable materials are used as a scaffold [poly(glycolic acid) (PGA), silk, and collagen]. Also the use of various cell lines is described, such as ACL and medial collateral ligament fibroblasts, bone marrow stromal cells (BMSCs), and tenocytes. All these studies show promising results in the attachment, proliferation, and differentiation of these cells when seeded onto the scaffolds.

The appropriate cell type for ligament tissue engineering must show enhanced proliferation and production of an appropriate extracellular matrix and must be able to survive in an intraarticular environment in the patient's knee. ACL fibroblasts are cells specialized in producing all ligament constituents and maintaining the ligament tissue in the appropriate conformation. However, it is well known that the anterior cruciate ligament has poor healing capacities in contrast to other ligaments, such as the medial collateral ligament. This deficient repair could be due to several intrinsic properties of the cells, as responsiveness to growth factors,^{17,18} adhesion strength,^{19–21} proliferation, and migration rate were reported to be lower compared with other cell types.^{19–26} Bellincampi *et al.* demonstrated the survival of skin fibroblasts in an intraarticular environment to be superior over ACL fibroblasts,¹⁰ making this cell source also a potential donor cell for our engineered ligament. BMSCs are cells that have been shown to be capable of differentiating toward various types of connective tissue, such as bone and cartilage^{27–29} and also ligament fibroblasts.^{30–32} Although BMSCs are used in *in vivo* experiments for tendon repair,^{11,13} no data are available on the behavior of BMSCs in ligament repair in an intraarticular synovial environment. The purpose of this study was to compare ACL and skin fibroblasts and BMSCs with regard to cell proliferation and differentiation (matrix synthesis) on three-dimensional bipolymer scaffolds, in order to determine the optimal cell source for tissue engineering of ligaments. The cells were seeded at various densities onto braided poly(L-lactide/glycolide) scaffolds to study the effect of initial seeding density on proliferation and matrix synthesis.

MATERIALS AND METHODS

Design

BMSCs, skin fibroblasts, and ACL fibroblasts were all isolated from one adult Dutch milk goat. After culture expansion, the three different cell types were seeded onto

a scaffold made of braided Panacryl. For each cell type, five different seeding densities were used: 1×10^4 , 3×10^4 , 1×10^5 , 3×10^5 , and 1×10^6 cells per scaffold. All samples were analyzed for various proliferation and differentiation parameters on day 1, day 6, and day 12 (block staining, environmental scanning electronic microscopy, DNA fluorometry, total collagen biochemistry, histology, and immunohistochemistry). Each quantitative analysis was performed in triplicate.

Cell isolation and expansion

Skin fibroblasts. The skin of the proximal left hind leg was retrieved, using a dermatome, after shaving the skin. The skin was subsequently rinsed three times with phosphate-buffered saline (PBS; GIBCO, Grand Island, NY), transferred to a petri dish containing a PBS solution with 0.25% dispase II (Roche, Mannheim, Germany), and stored at 4°C for 17 h. Next, the dermis was split from the epidermis, using a forceps. The dermis was minced and transferred to 15 mL of 0.25% dispase II–0.75% collagenase A solution (Roche) and incubated on an XYZ shaker (Polymax; Heidolph Instruments, Schwabach, Germany) at 37°C for 3 h. Subsequently the suspension was passed through a filter chamber (200- μ m pore size; NTBI, Emmercompascuum, The Netherlands).

ACL fibroblasts. Left and right anterior cruciate ligaments were excised under sterile conditions and minced. The pieces were suspended in a 0.15% collagenase II solution (Worthington Biochemical, Lakewood, NJ) and incubated on an XYZ shaker at 37°C for 20 h. Subsequently the suspension was filtered through a cell strainer (Falcon, 100- μ m pore size; BD Biosciences Discovery Labware, Bedford, MA).

The ACL and dermis suspensions were expanded in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO) penicillin–streptomycin (100 U/mL each), and L-glutamine (580 mg/L), a culture medium that has been found to be optimal for cells of the fibroblast lineage. Cells were cultured until confluence, with medium changes performed three times per week.

Bone marrow stromal cells. Bone marrow was aspirated from the distal and proximal left femur under sterile conditions. The aspirate was cultured until confluence in expansion medium. Although previously no significant differences between α -MEM and DMEM were observed with respect to proliferation and differentiation of subcultured human BMSCs,³³ we routinely used α -MEM (GIBCO) for expansion of BMSCs, supplemented with 10% fetal bovine serum (GIBCO), L-glutamine (2 mM; GIBCO), ascorbic acid (0.2 mM; Sigma), and penicillin–streptomycin (each 100 U/mL; GIBCO). The cul-

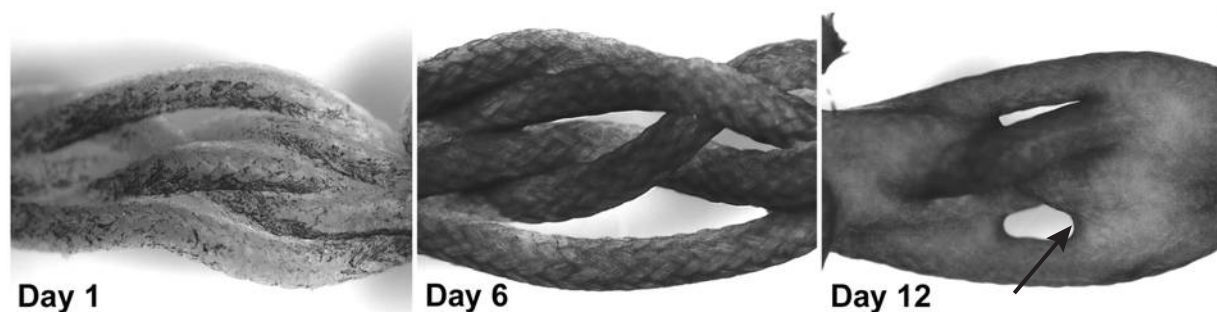


FIG. 1. Effect of culture period on cell content and matrix deposition. Methylene blue staining of scaffolds seeded with 100,000 BMSCs (original magnification, $\times 5$). Increased accumulation of cells and matrix is observed with time. Similar effects of culture period were observed for scaffolds seeded with skin and ACL fibroblasts. Arrow: tissue bridging the multifilaments.

ture medium was renewed three times per week, during which nonadherent cells were removed from the culture flask.

Scaffolds

The degradable scaffolds were made of braided Panacryl (size 0; Ethicon, Somerville, NJ). Panacryl is composed of 11- μm poly(L-lactide/glycolide) fibers, braided in bundles to a multifilament of $\pm 450\ \mu\text{m}$ and coated with 90% caprolactone and 10% glycolide. Five strands were braided together and pieces approximately

1 cm in length were knotted at the ends with Panacryl. For resterilization after handling, the braided scaffolds were autoclaved at 121°C for 20 min. On autoclaving, the scaffolds appeared to have slightly diminished in size. The scaffolds were subsequently incubated in medium supplemented with 10% FBS the night before seeding.

Seeding and three-dimensional culture

As proliferation and differentiation have been demonstrated not to differ between passage 3 and 4 of human BMSCs³⁴ and skin fibroblasts,³⁵ BMSCs were cultured

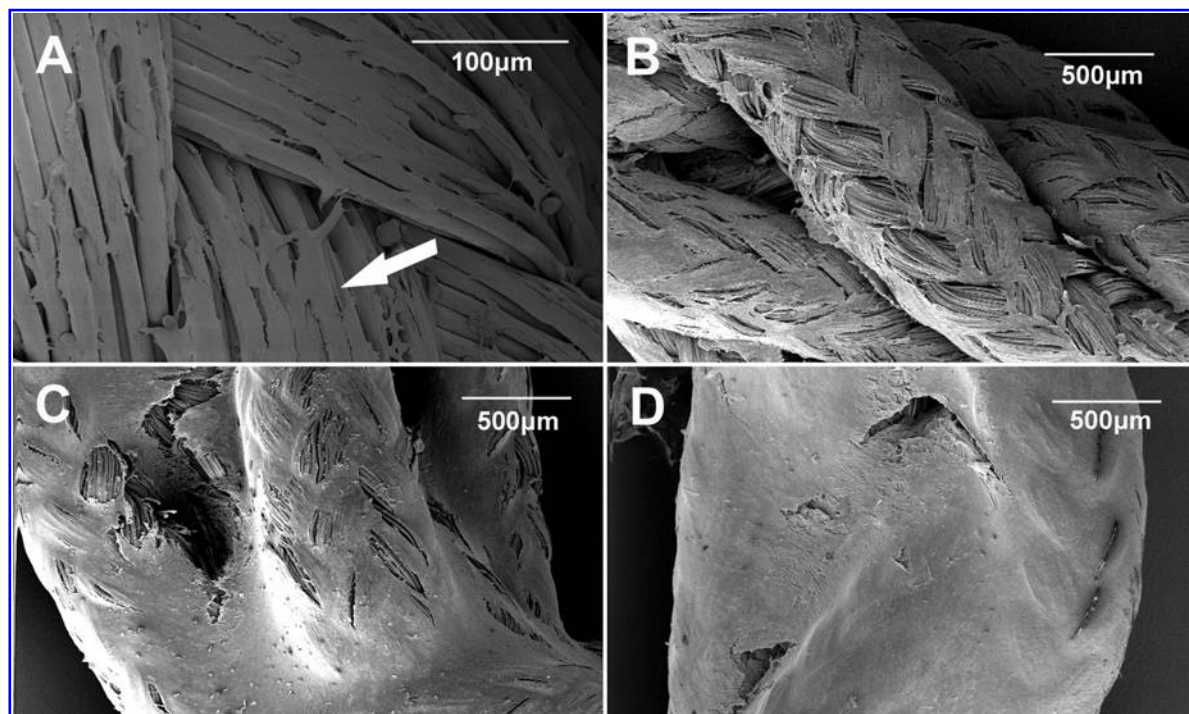


FIG. 2. Matrix deposition by BMSCs, ACL, and skin fibroblasts. (A) Alignment of BMSCs with monofilaments on day 1. Scanning electron micrograph of scaffolds seeded and cultured for 12 days with ACL fibroblasts (B), skin fibroblasts (arrow) (C), and (D) BMSCs. Covering of scaffolds with cells and matrix is visibly most complete with BMSCs (scaffolds all seeded with 100,000 cells).

to passage 3 and the skin and the ACL fibroblasts were seeded at passage 4 in order to be able to seed all cells at the same time. The cultured cells were trypsinized, counted, and resuspended. ACL and skin fibroblasts were resuspended in DMEM, to which, in addition to the supplements of the expansion mix, ascorbic acid (0.2 mM) and 10% FBS had been added. BMSCs were resuspended in the same medium as used for expansion, α -MEM containing 10% FBS and 0.2 mM ascorbic acid. Aliquots of 2 mL containing 1×10^4 , 3×10^4 , 1×10^5 , 3×10^5 , or 1×10^6 cells were added to 2-mL tubes (Eppendorf, Hamburg, Germany), each containing one scaffold. The tubes were sealed with a hydrophilic filter (Eppendorf) and placed on a roller bank (1 rpm, low profile roller; New Brunswick Scientific, Edison, NJ) at 37°C and 5% CO₂. After 24 h, the scaffolds were removed from the tubes and transferred to replica well plates (Sterilin, Stone, Staffordshire, UK) containing 2 mL of medium in each well. These plates were cultured on an XYZ shaker

at 30 rpm (37°C, 5% CO₂). Medium was changed three times per week.

Outcome parameters

Cell attachment. Methylene blue block staining was performed to visualize cell attachment. The samples were carefully rinsed with PBS and fixed with 1.5% glutaraldehyde in 0.14 M cacodylate buffer overnight. The scaffolds were stained with methylene blue and subsequently placed under a stereomicroscope (SM2-10A; Nikon, Tokyo, Japan).

Matrix structure. Environmental scanning electron microscopy (ESEM; Philips, Eindhoven, The Netherlands) was used to qualitatively evaluate matrix synthesis. Using this technique, the structure and the produced matrix can be visualized. To prepare for ESEM, the scaffolds that were previously used for block staining were dehydrated, critical point dried, and gold sputter coated.

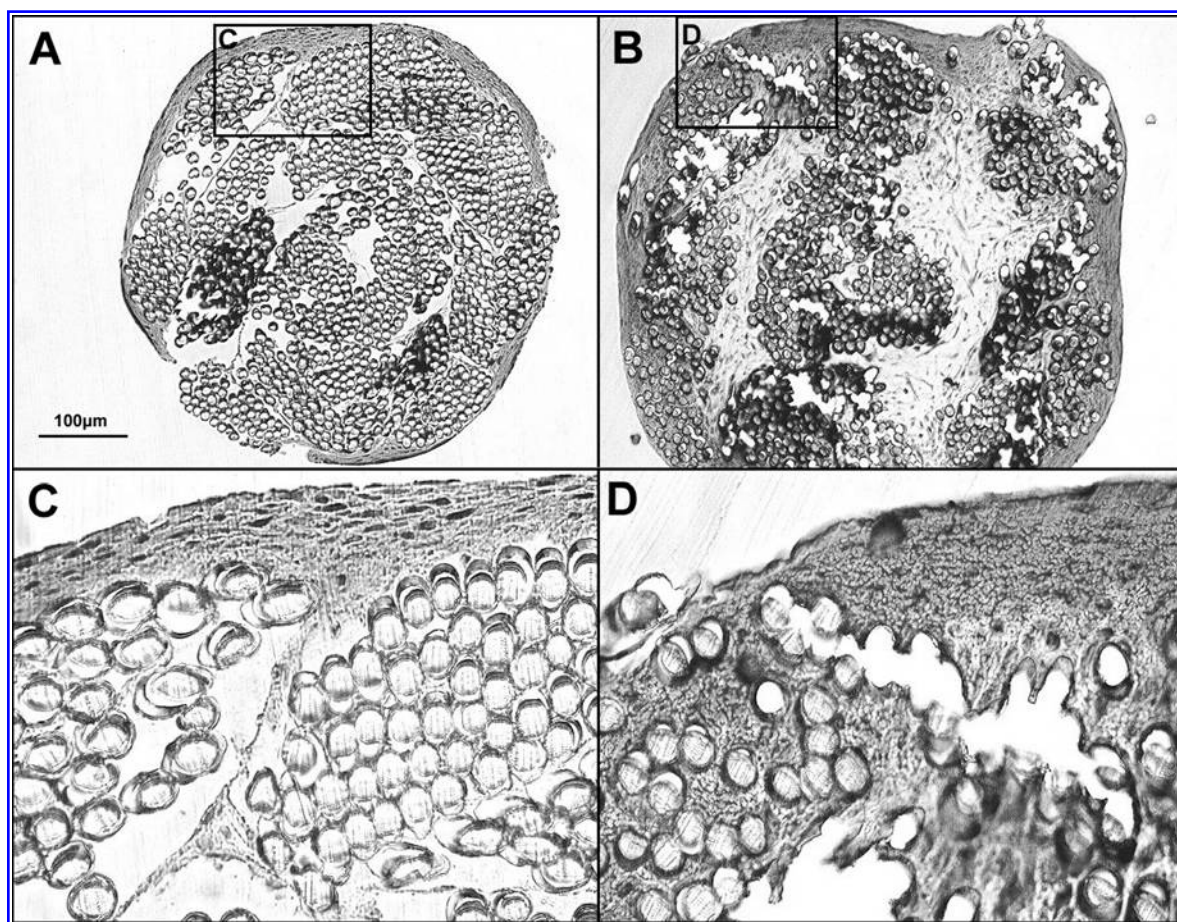


FIG. 3. Histology of scaffolds seeded with 300,000 BMSCs on day 12. H&E staining of cross-sections showing (A and C) cells and tissue visible only on the outside of the scaffold and (B and D) cells and tissue present both on the outside and at the center of the scaffold in areas with a more open structure between the monofilaments.

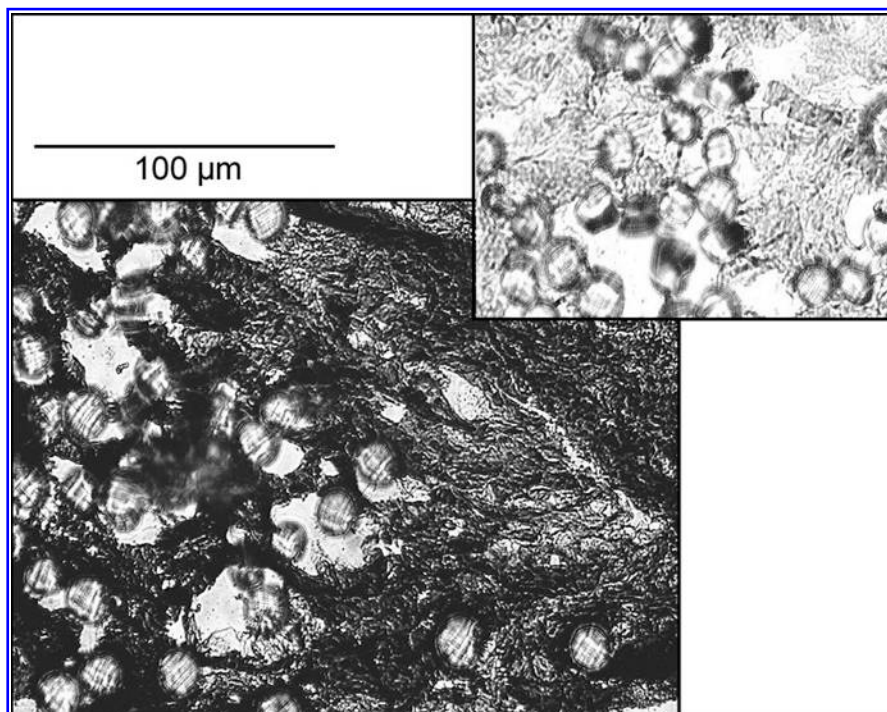


FIG. 4. Immunohistochemistry for collagen type I. Collagen type I staining was demonstrated for all cell-seeded scaffolds independent of the cell type seeded (shown: scaffold seeded with 100,000 skin fibroblasts on day 12. *Inset:* Negative control).

Cell proliferation. Cell proliferation was assessed by fluorometric quantification of DNA, using the CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR). After rinsing with PBS, all samples were digested in a 1.0-mg/mL proteinase K solution (Sigma) for 16 h at 56°C, after which the samples were treated according to the manufacturer's instructions. The fluorometric analysis was done at 560 nm with a luminescence spectrometer (PerkinElmer, Buckinghamshire, UK).

Collagen quantification. As a parameter for matrix synthesis, we determined the total amount of collagen, using the hydroxyproline assay described by Brown *et al.*³⁶ Briefly, aliquots of 100 μ L of the proteinase K digests used for the DNA assay were hydrolyzed overnight with 100 μ L of 12 M HCl at 110°C in a heating block (Techne, Cambridge, UK). Fluid was removed by nitrogen evaporation (evaporator; Pierce, Rockford, IL). The hydrolysates were dissolved in demineralized water. The solutions were oxidized with Chloramine-T (Sigma) followed by the Ehrlich reaction, adding *p*-methyl-amino-benzaldehyde (Sigma) dissolved in a propanol-perchloric acid solution (Merck, Darmstadt, Germany). Absorbance was measured at 570 nm with a microplate reader (Biokinetics reader; Bio-Tek Instruments, Winooski, VT). Collagen content was calculated

by assuming 14% as the content of hydroxyproline in collagen of ligaments.³⁷

Histology and immunohistochemistry. For histology, the scaffolds were carefully rinsed with PBS, fixed with 1.5% glutaraldehyde in 0.14 M cacodylate buffer

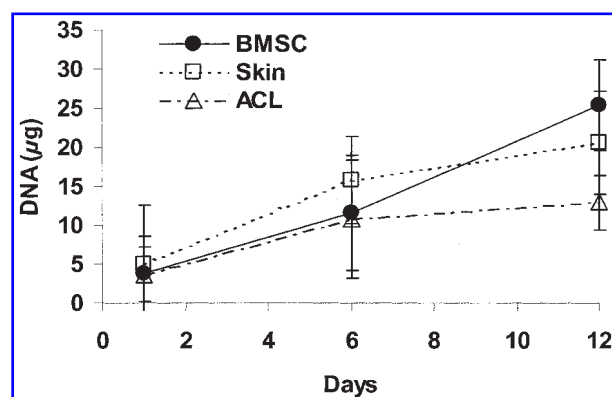


FIG. 5. Increase in DNA content during culture. During the culture period of 12 days, an increase in total DNA per scaffold was seen for all cell types (pooled seeding densities, $n = 15$). On day 12, the scaffolds seeded with BMSCs showed the highest DNA content ($p < 0.01$).

TABLE 1. TOTAL DNA, COLLAGEN, AND COLLAGEN/DNA ON DAY 12^a

Cell type	DNA (μg)		Total collagen (μg)		Collagen/DNA	
	Mean	SD	Mean	SD	Mean	SD
ACL	12.97	3.49	18.02	5.33	1.43	0.40
Skin	20.57	6.56	74.99	46.93	3.38	1.68
BMSCs	25.40	5.77	114.01	59.45	4.26	1.80

^aThe scaffolds seeded with BMSCs showed a higher DNA content as compared with the other cell types ($p < 0.01$; pooled seeding densities, $n = 15$). Differences between the various cell types were statistically significant ($p < 0.01$).

overnight, dehydrated and embedded in glycol methacrylate (GMA), followed by cutting with a microtome and hematoxylin and eosin (H&E) staining. For immunohistochemistry of collagen type I, the scaffolds were rinsed with PBS, embedded in Tissue-Tek O.T.C. compound (Sakura Finetek USA, Torrance, CA), and stored at -80°C . The samples were sectioned and stained for collagen type I (Oncogene Research Products/EMD Biosciences, San Diego, CA), using a 1:200 dilution and pretreatment with proteinase K solution for 10 min (Dako Cytomation, Carpinteria, CA). Counterstaining was done with hematoxylin. Both immunosections and histological sections were analyzed by light microscopy (Eclipse E600; Nikon).

Statistical analysis

Differences in cell proliferation and collagen synthesis were assessed by analysis of variance (ANOVA). Multiple paired comparisons of the factors cell type, seed-

ing density, and day were calculated with a Bonferroni correction. Analyses were carried out with SPSS version 10.0 (SPSS, Chicago, IL). As univariate analysis revealed that there was no interaction between cell type and seeding density, data derived from scaffolds seeded at various cell densities were pooled per cell type to analyze the overall difference in collagen and DNA content between the cell types. A p value of <0.05 was considered statistically significant.

RESULTS

Cell attachment, proliferation, and matrix formation

All cell types readily attached to the neoligament scaffold as observed by methylene blue block staining on day 1 (Fig. 1). At this time, the cells flattened and began to proliferate. As illustrated by ESEM (Fig. 2D), the cells were aligned along the axis of the monofilaments. By day 6, the cells fully surrounded the multifilaments, and the BMSCs and skin fibroblasts seeded at 1×10^6 even bridged the gaps between the individual multifilaments. This bridging was present for all cell types on day 12.

Also by light microscopy, cells and matrix were shown to be present on all outer surfaces of the scaffolds (Fig. 3). On these outer surfaces of the scaffolds, the cells were mainly elongated in shape and densely packed in layers, whereas more toward the middle of the scaffold the cells were more ovoid in shape and the tissue was less dense. Hardly any cell infiltration and matrix production were seen in the center of the multifilaments, but in areas with a more open structure between the monofilaments, such as in the tip of the sutures, cells and tissue were present inside the scaffold (Fig. 3B and D).

The produced extracellular matrix contained fibrous structures (diameters between 15 and 130 nm, measured by ESEM) and the amount increased in time for each cell type, as indicated by ESEM (data not shown). Immunohistochemistry demonstrated the presence of collagen type I for each cell type on day 6 and day 12 (Fig. 4).

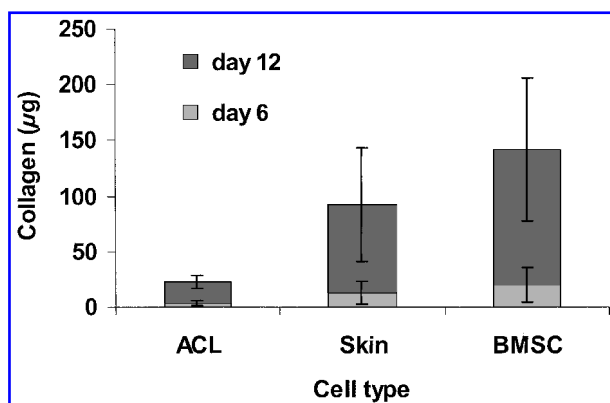


FIG. 6. Collagen production on days 6 and 12. On day 12, total collagen content (in micrograms) was highest for the scaffolds seeded with BMSCs as compared with the other cell types (seeding densities are pooled to illustrate the effect of cell type on collagen production, $n = 15$). The increase in collagen content was statistically significant between day 6 and day 12 for each cell type.

TABLE 2. COLLAGEN AND COLLAGEN/DNA, PER SEEDING DENSITY ON DAY 12^a

Seeding density	Comparison	Collagen (μg) (mean $\pm$ SD)	Collagen/DNA (mean $\pm$ SD)
10,000	ACL–skin	13.0 $\pm$ 4.2 < 18.6 $\pm$ 1.4	1.5 $\pm$ 0.8 > 1.3 $\pm$ 0.1
	ACL–BMSCs	13.0 $\pm$ 4.2 < 32.5 $\pm$ 3.7	1.5 $\pm$ 0.8 < 1.9 $\pm$ 0.1
	Skin–BMSCs	18.6 $\pm$ 1.4 < 32.5 $\pm$ 3.7	1.3 $\pm$ 0.1 < 1.9 $\pm$ 0.1
30,000	ACL–skin	17.7 $\pm$ 3.9 < 28.3 $\pm$ 9.8	1.5 $\pm$ 0.2 < 2.1 $\pm$ 0.9
	ACL–BMSCs	17.7 $\pm$ 3.9 < 69.0 $\pm$ 7.4 ^b	1.5 $\pm$ 0.2 < 3.0 $\pm$ 0.5
	Skin–BMSCs	28.3 $\pm$ 9.8 < 69.0 $\pm$ 7.4 ^c	2.1 $\pm$ 0.9 < 3.0 $\pm$ 0.5
100,000	ACL–skin	17.6 $\pm$ 8.3 < 86.8 $\pm$ 17.5 ^b	1.5 $\pm$ 0.3 < 4.6 $\pm$ 1.5 ^b
	ACL–BMSCs	17.6 $\pm$ 8.3 < 128.9 $\pm$ 19.7	1.5 $\pm$ 0.3 < 4.8 $\pm$ 0.6 ^b
	Skin–BMSCs	86.8 $\pm$ 17.5 < 128.9 $\pm$ 19.7 ^c	4.6 $\pm$ 1.5 < 4.8 $\pm$ 0.6
300,000	ACL–skin	23.6 $\pm$ 0.4 < 122.9 $\pm$ 22.6 ^b	1.5 $\pm$ 0.3 < 4.9 $\pm$ 1.1 ^b
	ACL–BMSCs	23.6 $\pm$ 0.4 < 161.0 $\pm$ 64.4 ^b	1.5 $\pm$ 0.3 < 5.6 $\pm$ 1.2 ^b
	Skin–BMSCs	122.9 $\pm$ 22.6 < 161.0 $\pm$ 64.4 ^d	4.9 $\pm$ 1.1 < 5.6 $\pm$ 1.2
1,000,000	ACL–skin	18.2 $\pm$ 3.7 < 118.3 $\pm$ 1.0 ^b	1.1 $\pm$ 0.3 < 4.0 $\pm$ 0.4 ^b
	ACL–BMSCs	18.2 $\pm$ 3.7 < 178.7 $\pm$ 19.4 ^b	1.1 $\pm$ 0.3 < 6.0 $\pm$ 1.4 ^b
	Skin–BMSCs	118.3 $\pm$ 1.0 < 178.7 $\pm$ 19.4 ^b	4.0 $\pm$ 0.4 < 6.0 $\pm$ 1.4

^aComparison of collagen content for the various cell types and seeding densities demonstrated a statistically significant higher collagen content for scaffolds seeded with BMSCs as compared with the other cell types at seeding densities of 300,000 and 1,000,000. Scaffolds seeded with BMSCs and skin fibroblasts yielded statistically significantly higher values of collagen per DNA at the three highest seeding densities.

^b $p < 0.01$.

^c $p < 0.02$.

^d $p < 0.05$.

Effect of cell type on proliferation and matrix production

Proliferation. Analysis of DNA content revealed no significant interaction of cell type with seeding density, and therefore all seeding densities were pooled to analyze the overall difference between cell types.

The increase in cell number over time for each cell type, as suggested by methylene blue staining, was confirmed by the DNA assay (Fig. 5). On day 12, a statistically significant difference was observed, the scaffolds seeded with BMSCs displaying the highest DNA content ($p < 0.01$; Table 1). The increase in DNA for the BMSCs was highest between day 6 and day 12. In contrast, the DNA content of scaffolds seeded with ACL and skin fibroblasts displayed the highest increase between day 1 and day 6 (Fig. 5).

Matrix production. ESEM evaluation indicated that the BMSCs had produced the highest amount of matrix on day 12 (Fig. 2B–D). This was confirmed by measuring the hydroxyproline content of scaffolds as a parameter for collagen (matrix) production. As the analysis of collagen content and collagen per DNA after 12 days of culture showed a significant interaction of cell type with seeding density, seeding densities were not pooled. Statistical analysis of the difference between the cell types with respect to collagen production and collagen per

DNA after 12 days was performed per seeding density. For all cell types, collagen content of the scaffolds increased with culture time (Fig. 6). The increase in collagen content over time was statistically significant for each cell type ($p < 0.05$, except for the seeding density of 1×10^4 cells per scaffold for both the BMSCs and the skin fibroblasts), with the increase in BMSCs and skin fibro-

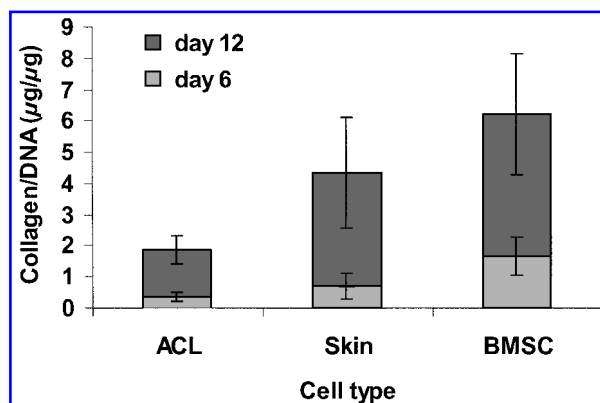


FIG. 7. Total collagen per microgram of DNA on days 6 and 12. Collagen production per microgram of DNA on day 12 was highest for the scaffolds seeded with BMSCs when compared with ACL and skin fibroblasts (seeding densities are pooled to illustrate the effect of cell type on collagen production per microgram of DNA, $n = 15$).

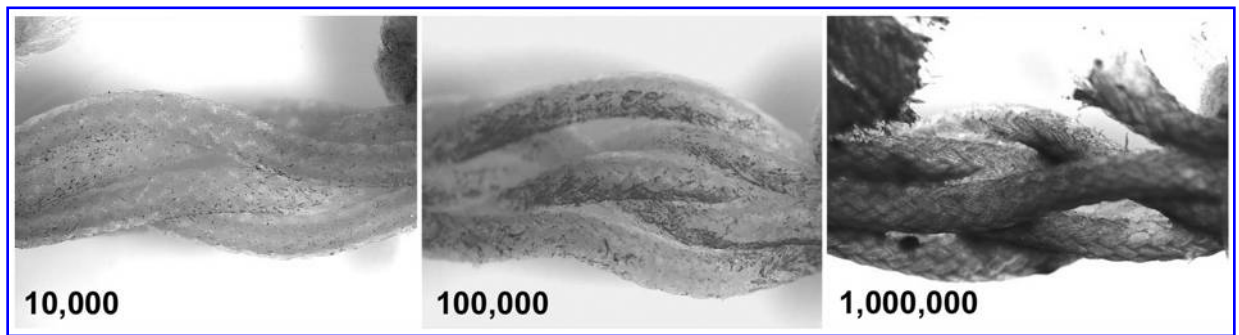


FIG. 8. Effect of seeding density on cell content on day 1. Methylene blue staining of scaffolds seeded with BMSCs (original magnification, $\times 5$). Increased accumulation of cells is observed with increasing seeding densities. Similar effects of seeding density were observed for scaffolds seeded with skin and ACL fibroblasts.

blasts being higher than the increase in ACL fibroblasts. On day 12, collagen content was highest for the scaffolds seeded with BMSCs as compared with other cell types (Fig. 6 and Table 2). This difference was significant for the two highest seeding densities ($p < 0.05$; Table 2).

When expressed in terms of collagen produced per microgram of DNA, the scaffolds seeded with BMSCs and skin fibroblasts showed a higher level of collagen production per microgram of DNA on day 12 as compared with ACL fibroblasts (Fig. 7, statistically significant only for the three highest seeding densities, $p < 0.01$; Table 2). Between day 6 and day 12, an increase in collagen production per microgram of DNA was found, which was clearly higher for the BMSCs and the skin fibroblasts as compared with the ACL fibroblasts ($p < 0.05$, except for a seeding density of 10,000 per scaffold for the BMSCs).

Effect of seeding density on proliferation and matrix synthesis

The number of cells that attached to the scaffold increased with increasing seeding density (Fig. 8). For the ACL fibroblasts, there were no statistically significant differences found in DNA and collagen production between the various seeding densities on day 12 (Fig. 9A–C). On day 12, DNA content showed a more pronounced increase with increasing seeding density for the scaffolds seeded with BMSCs and skin fibroblasts (Fig. 9A). Collagen content and collagen per DNA also showed an increase with increasing seeding density (Fig. 9B and C), with the three highest seeding densities showing the highest amounts.

DISCUSSION

In the current study, several cell types were compared for their capacity to enhance ligament formation on braided biodegradable scaffolds. Previous studies on tissue engineering of ligaments describe the use of various cell lines, such as ACL fibroblasts, medial collateral lig-

ament (MCL) fibroblasts, skin fibroblasts, BMSCs, and tenocytes.^{8–16,38} The present study is, to the best of our knowledge, the first to compare three different cell types with regard to two important parameters in ligament tissue engineering: *in vitro* proliferation and matrix production.

Seeding of the Panacryl scaffolds with ACL, skin, and bone marrow stromal stem cells resulted in attachment and alignment along the longitudinal axis of the scaffolds. With time a distinct proliferation and matrix synthesis, as reflected by the final collagen content, was shown for all cell types. However, clear differences were noted between the cell types with respect to proliferation, total collagen production, and collagen production per cell, as reflected by DNA content. Proliferative activity of the BMSCs was highest, whereas skin fibroblasts proliferated more readily than ACL cells. Also, the final collagen content was highest for the scaffolds seeded with BMSCs and lowest in those seeded with ACL fibroblasts. When collagen production was expressed per cell, there was no difference between skin fibroblasts and BMSCs, but the ACL cells had clearly generated less collagen, thus indicating that the difference between skin fibroblasts and BMSCs in final collagen content was mainly due to a higher cell number at the end of the culture period. Both for skin fibroblasts and BMSCs, seeding density correlated positively with final collagen content, although densities beyond 3×10^5 did not seem to bring about more collagen deposition. For ACL fibroblasts an increase was indicated, but was so low that no statistical significance could be attained.

The presence of collagen type I in the produced matrix was an important finding. Native ligaments and tendons typically consist of 90% collagen type I and less than 10% collagen type III. The fibrous structures observed with ESEM probably are collagen fibrils that were formed out of collagen microfibrils produced by the cells. The collagen dimensions measured in this study are in the range of those described elsewhere (between 20 and 500 nm).³⁹

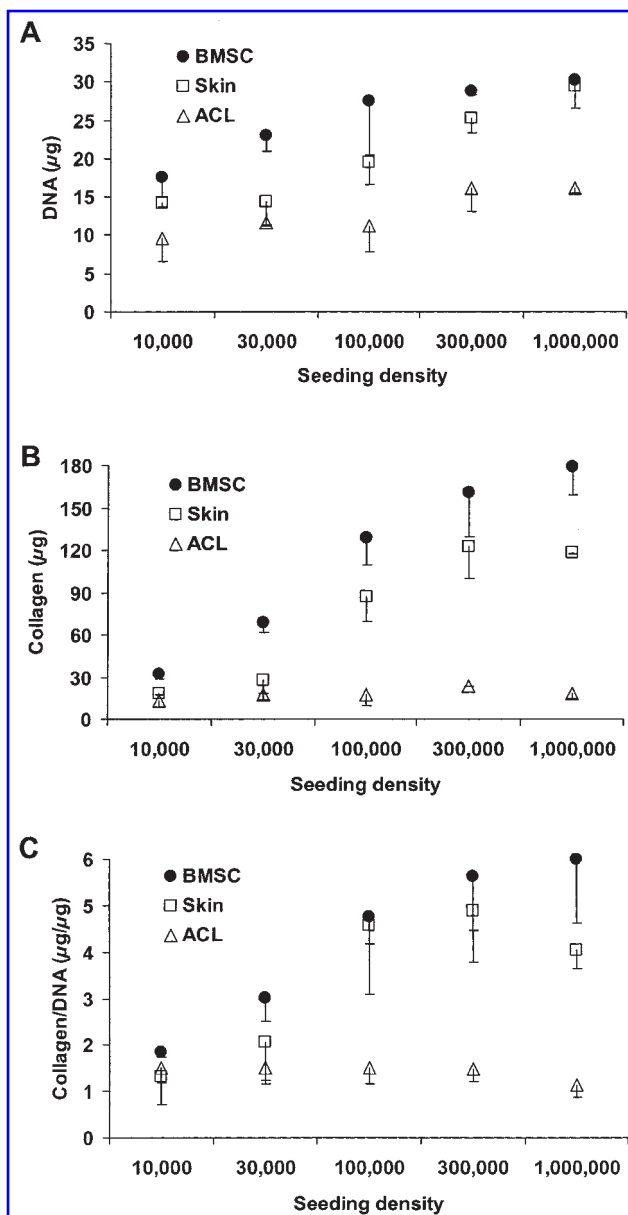


FIG. 9. Effect of seeding density on DNA content (A), collagen production (B), and collagen/DNA (C) on day 12. For the scaffolds seeded with ACL cells, the yield of DNA and collagen on day 12 did not significantly differ between seeding densities. For the scaffolds seeded with BMSCs and skin fibroblasts, an increase in DNA, collagen, and collagen/DNA was found with increasing seeding density ($n = 3$). The three highest seeding densities showed the highest amounts of collagen and collagen per DNA for the BMSCs and the skin fibroblasts ($n = 3$).

For cell-based tissue engineering, the choice of cell type is crucial and is influenced by various considerations. For ligament tissue engineering, ACL fibroblasts initially would seem the optimal candidate, as they are highly specialized in the production of ligament tissue. However, in line with previous findings,^{9,17,22} the pres-

ent study shows that ACL proliferation and matrix production lagged far behind the other two cell types, even when seeded at higher densities. In contrast, both skin cells and BMSCs readily proliferated and deposited collagen onto the scaffolds. As the proliferative activity of the BMSCs was in general higher than that of skin fibroblasts, total matrix production was optimal with the former cell type. It is noteworthy that, in contrast to ACL fibroblasts, both skin fibroblasts and BMSCs display an increase in collagen production per cell with increasing seeding density, indicating that the balance between proliferation and matrix production might be dependent on the amount of cells already present. A similar effect is reported by Carrier *et al.*, who describe an increase in the fraction of differentiated cardiac myocytes with the number of cells seeded.⁴⁰ In contrast, the ACL cells produced the same amount of collagen per cell irrespective of initial seeding density, possibly because of their highly differentiated state. Despite the fact that the ACL and skin cells are in theory the same cell type, there is clearly a significant difference in their potency to adapt to different circumstances. Skin fibroblasts are known to have great healing potential and have already been shown to survive in an intraarticular environment.¹⁰

From the present data it appears that both BMSCs and possibly to a lesser extent skin fibroblasts possess the appropriate characteristics for application in ligament tissue engineering, whereas ACL fibroblasts seem of limited use. Moreover, in a clinical setting, BMSCs and skin fibroblasts are easily retrieved by bone marrow biopsy and a split skin graft, respectively, conferring an additional advantage to the use of these cells over ACL fibroblasts. ACL fibroblasts are more difficult to obtain, because this requires an arthroscopy in the already injured knee. As the data are based on one donor, a broad generalization is not yet in view. However, our experiences and those of other researchers with goat cells indicate that interdonor variation is small in terms of cell proliferation⁴¹ and cartilage formation and osteogenesis *in vivo*.⁴²

We conclude that bone marrow stromal cells, and possibly skin fibroblasts, promise to be the cell type of choice in ligament tissue engineering and warrant further investigations focusing on other relevant parameters, such as the survival of the cells in an intraarticular environment and the mechanical properties of the produced matrix.

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