Characterization of the structure of rabbit anterior cruciate ligament and its stem/progenitor cells

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Abstract
Background: It is known that anterior cruciate ligament (ACL) of the knee joint is prone to injuries with poor healing potential. The healing capacity of a tissue-like ACL is dependent on its structural components and the properties of the stem cells (SCs). Therefore, this study aimed to characterize the structure of ACL tissue and the properties of the SCs derived from the tissue components.

Methods: The tissue structure of rabbit ACL was determined using a scanning electron microscope, hematoxylin and eosin, and immunohistochemical staining. The biological properties of SCs derived from the structural components of ACL were studied by colony formation, cell proliferation assay, SC marker expression and collagen exhibition, and multidifferentiation potential.

Results: The two distinct components of ACL are classified as sheath and core, which possess differential properties in terms of collagen type, organization, and presence of blood vessels. The sheath tissue contains vascular SCs and the core tissue contains ligamentous SCs, respectively. The two types of SCs differ in clonogenicity, proliferation, and multidifferentiation potential.

Conclusion: This study shows that ACL consists of sheath and core tissues, which contain sheath and core SCs with distinctive biological properties. These findings highlight the need for use of both sheath and core SCs to promote the repair of the complex structure of injured ACL.

KEYWORDS
anterior cruciate ligament (ACL), core, differentiation, sheath, stem cells (SCs)

1 | BACKGROUND

Anterior cruciate ligament (ACL) plays an important role in controlling the movements of the knee joint. As such, ACL is subject to large mechanical loads and consequently frequently injured, particularly in the athletic settings.1,2 In the United States alone, around 350 000 reconstructive ACL surgeries are performed annually and the cost for the acute care of the ACL injuries is around $6 billion.3 ACL injuries may lead to a spectrum of secondary symptoms, including knee laxity, movement dysfunction, meniscus, and cartilage damage, and even the development of secondary symptoms.

Abbreviations: ACL, anterior cruciate ligament; ACL-C, ACL core; ACL-S, ACL sheath; Col II, collagen type II; H&E, hematoxylin and eosin; MCL, medial collateral ligament; MSC, mesenchymal stem cells; NS, nucleostemin; Oct-4, octamer-binding transcription factor 4; SC, stem cell; SEM, scanning electron microscopy.
degenerative joint disease. Despite the high prevalence of ACL injuries, no effective treatment is currently available in clinics because the properties of the ACL tissues and its cells are still incompletely understood. Surgical treatment is the most widely applied procedure, meaning that autogenous and allograft tendon grafts are used in ACL reconstruction. However, failure of such grafts is a major clinical problem. Therefore, it is highly desirable to devise biological augmentation strategies to repair injured ACL. However, it is known that ACL has poor healing potential after injury. Such poor healing capacity may be related to the complex structure of ACL as well as its stem cells (SCs), which are known to play a key role in the repair of injured tissues.

Mesenchymal stem cells (MSCs) from human ACL have been isolated previously. Our previous study has characterized adult SCs from human ACL possessing clonogenicity, self-renewal, SC marker expression (oc-tamer-binding transcription factor 4 [Oct-4], nucleostemin [NS]), and multidifferentiation potential. In addition, another study suggested that human ACL septum region contains a population of vascular-derived SCs that are CD34-positive and CD146-positive, which exhibit multi-lineage differentiation potential, including the capacity to undergo chondrogenesis, osteogenesis, adipogenesis, and endotheliogenesis. However, these studies have isolated a mixture of SCs from the whole ACL as a single unit.

Therefore, in this study our aim was twofold: the first was to characterize the structure of rabbit ACL. The detailed structure of ACL may allow us to better understand the complexity of the healing process in injured ACL. The second was to compare the biological properties of SCs derived from the structural components of ACL, which may enable us to develop new SC therapy to promote healing of injured ACL. The reason for using rabbit ACL is that it is a common model in the study of ACL biomechanics and biology as well as in surgical ACL reconstruction in human patients. We report that rabbit ACL comprises two distinct types of tissues, that is, loose sheath and dense core, with differential structural properties, and SCs from those tissues display differential biological properties.

2 | METHODS

2.1 | Tissue samples and ethics statement

The ACL tissue samples were obtained from six New Zealand white rabbits (5-month-old, female) right after sacrifice. The investigation was conducted in accordance with ethical standards and according to the Declaration of Helsinki and according to national and international guidelines. The protocol for use of the rabbits was approved by the IACUC of the University of Pittsburgh.

2.2 | Characterization of rabbit ACL using a scanning electron microscope

Visualization and imaging of rabbit ACL were performed using a scanning electron microscope (SEM). Rabbit ACL was fixed with 3% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer containing 1% sucrose and then incubated in 1% osmium tetroxide for another 30 minutes. The fixed samples were treated with 50%, 60%, 70%, 80%, 90%, and 100% ethanol for 15 minutes each for dehydration. The samples were treated with a critical point drying instrument and finally were coated with gold/palladium using a sputter coater. The structure of ACL was examined under a microscope (JEOL JSM-6510, Peabody, MA) with an accelerating voltage of 3 kV.

2.3 | Hematoxylin-eosin and immunohistochemical staining

ACL tissue samples were fixed in 4% paraformaldehyde for 48 hours at 4°C. The fixed tissues were embedded in paraffin and sectioned parallel to the longitudinal axis of the ACL with a thickness of 5 μm. These sections were deparaffinized and rehydrated for hematoxylin and eosin (H&E) staining according to the standard protocol. The stained sections were examined under light microscopy (Nikon eclipse, TE2000-U). For CD105, CD31, CD34, tenascin C, collagen type I (col I), and col IV immunohistochemical staining, the deparaffinized and rehydrated sections were incubated with 0.05% trypsin at 37°C for 30 minutes for antigen retrieval treatment. The treated sections were washed with phosphate-buffered saline (PBS) twice for 2 minutes each and reacted with goat anti-CD105 (1:350; Novus Biologicals, Littleton, CO), mouse anti-CD31 (1:350; Novus Biologicals), rat anti-CD34 (1:350; Genetex, Irvine, CA), mouse anti-tenascin C antibody (1:300; Abcam, Cambridge, MA), mouse anti-col I (1:1000; Abcam), and goat anti-col IV primary antibodies (1:1000; Abcam) overnight at 4°C. Sections were then washed twice in PBS for 5 minutes each before being incubated with a Cy3-conjugated donkey anti-goat immunoglobulin G (IgG) secondary antibody (1:1000; Millipore, Temecula, CA) for CD105 and col IV testing, Cy3-conjugated goat anti-mouse IgG secondary antibody (1:1000; Millipore) for CD31, tenasin C, and col I testing, and Cy3-conjugated goat anti-rat IgG second antibody for CD34 testing (1:1000; Millipore) for 2 hours at room temperature. Slides were washed again, counterstained with H33342 (5 μg/mL; Sigma, St Louis, MO) nuclear
stain for 2 minutes, and photographed with a fluorescence microscope (Nikon eclipse, TE2000-U).

2.4 Cell isolation and culture

The isolation of SCs from the sheath and core tissues of rabbit ACL was based on our published protocols. Briefly, the ACL sheath (ACL-S) was carefully separated from the ACL core (ACL-C) under the microscope (Keeler Ltd, Windsor, UK). The sheath and core tissues were minced into small pieces approximately 1 mm³. Wet tissue samples, each weighing 150 mg, were digested at 37°C for 1 hour in 1 mL of PBS containing 3 mg of collagenase type I and 4 mg of dispase. After centrifugation at 1500 g for 5 minutes, the cell pellets were resuspended with the Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (Gibco, Grand Island, NY) as a single cell suspension and then cultured in 60-mm petri dishes or T25 flasks in 5% CO₂ at 37°C.

2.5 Colony staining

At 10 days in culture, the cells from the ACL-S and ACL-C were rinsed twice with PBS for 5 minutes, fixed in 4% paraformaldehyde for 15 minutes, and stained with methyl violet for 30 minutes. After rinsing twice with PBS for 5 minutes, the colonies were visualized and counted under a microscope (Nikon eclipse, TE2000-U).

2.6 Cell proliferation assay

A cell proliferation assay was performed using a cell counting kit (CCK)-8 assay kit (Sigma). Briefly, ACL-S and ACL-C cells at passage 2 were seeded into 96-well plates at a density of 5 x 10³ cells/well. At days 1, 2, and 3 in culture, the medium was removed, and 10 μL/well CCK-8 solution with 100 μL culture medium was added to each well and incubated at 37°C for 2 hours. The absorbance value was measured with a microplate reader at a wavelength of 510 nm. At least three separate experiments were performed for the cell proliferation assay.

2.7 Immunohistochemical analysis of cell markers

The cultured cells at passage 2 were washed for 5 minutes twice in PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. For determining Oct-4 and NS expression, the fixed cells were treated with 0.1% of Triton X-100 for 30 minutes and washed with PBS for another three times. After blocking with 2% bovine serum albumin for 1 hour at room temperature, the cells were incubated with mouse anti-rabbit Oct-4 (1:250; Abcam), mouse anti-rabbit NS (1:200, Abcam), mouse anti-rabbit col I (1:300; Abcam), or goat anti-rabbit col IV (1:300; Abcam) primary antibodies overnight at 4°C. After washing the cells with PBS, a Cy3-conjugated goat anti-mouse secondary antibody (1:500; Invitrogen Molecular Probes, Carlsbad, CA) was applied at room temperature for 1 hour for detecting Oct-4, NS, and col I, and Cy3-conjugated donkey anti-goat secondary antibody was incubated at room temperature for 1 hour for detecting col IV. The cells were also counterstained with H33342 staining (Sigma). The stained cells were imaged using fluorescence microscopy (Nikon eclipse, TE2000-U). Each experiment was repeated at least three times.

2.8 Adipogenic differentiation assay

Cells (1 x 10⁵ per well) were cultured in 12-well plates for 14 days in StemPro adipogenesis differentiation cocktail (Life Technologies, Carlsbad, CA). The medium was changed every 3 days. Adipogenesis was evaluated using an Oil Red O staining assay, which detects intracellular lipid accumulation. At day 14, the cells were fixed using 4% paraformaldehyde for 1 hour at room temperature, washed with PBS three times, and finally incubated with a 0.36% Oil Red O solution (Millipore) for 1 hour, followed by washing three times with DDH₂O. The dye was extracted with 100% isopropanol and the absorbance was read at 490 nm. At least three separate experiments were performed for cell differentiation assays, including chondrogenic differentiation and osteogenic differentiation assays described below.

2.9 Chondrogenic differentiation assay

Cells (1 x 10⁵ per well) were cultured in 12-well plates for 14 days in StemPro chondrogenesis differentiation cocktail (Life Technologies). Chondrogenesis was detected using a Safranin O staining assay. The cells were fixed in ice-cold ethanol for 1 hour, rinsed with DDH₂O twice each for 5 minutes, and stained at room temperature for 30 minutes with Safranin O solution (Sigma). The dye was extracted with 100% isopropanol and the absorbance was read at 510 nm.

2.10 Osteogenic differentiation assay

Cells (1 x 10⁵ per well) were cultured in 12-well plates for 14 days in StemPro osteogenesis differentiation cocktail (Life Technologies). Then the cells were fixed in chilled 70% ethanol for 1 hour, rinsed with distilled water twice each for 5 minutes, and stained with Alizarin Red S (Millipore) at room temperature for 30 minutes. The dye was extracted...
with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich, St Louis, MO) in 0.1 M phosphate buffer (pH 7.0), and the absorbance was read at 550 nm.

2.11 | Statistical analysis

Data are expressed as means ± standard deviations. Comparisons between groups were performed with the two-tailed Student t test. Statistical significance was defined by the value of $P < 0.05$. Each experiment was performed at least three times.

3 | RESULTS

3.1 | The structure of rabbit ACL

The detailed structure and composition of rabbit ACL was evaluated by SEM, histochemical staining, and immunostaining. Both horizontal and cross-sections of ACL showed two distinct types of tissues, a loose network sheath with disorganized mesh collagen fibers and a dense core component with parallelly organized collagen fibers (Figure 1). H&E staining further confirmed that the

**FIGURE 1** Characterization of rabbit ACL structure by SEM. The left panels are horizontal section (H-section) views of the ACL, whereas the right panels are vertical section (V-section) of the ACL. A, The surface structure of rabbit ACL shows two distinct parts, an outer loose sheath tissue (yellow box) and dense core tissue (red box). B, Enlarged area of sheath (yellow box in A) with disorganized collagen fibers in a net mesh (red arrows). C, Enlarged area of core (red box in A) with well-organized collagen fibers (green arrows). D, Vertical section of the ACL shows a similar structure with two distinct parts, loose sheath (yellow box) and dense core (red box). E, Enlarged image of the yellow box in D shows loose network of fibers in sheath (red arrow). F, Enlarged image of the red box in D shows well-organized fibers in core (green arrows). Yellow bar: 50 µm; blue bar: 1 µm; red bar: 10 µm; and green bars: 25 nm. ACL, anterior cruciate ligament; SEM, scanning electron microscopy.
ACL has two different components, the loose sheath and the dense core tissue without a septum (Figure 2). The sheath area has disorganized collagen fibers forming a mesh, but with vessel-like structures (Figure 2A and 2B). On the other hand, the core area has dense organized fibers with the presence of a few vessels (Figure 2A and 2C). Further characterization was performed by immunostaining for CD105 (vascular pericyte marker), CD31, CD34 (vascular endothelial markers), tenascin C (ligament marker), col I (ligament marker), and col IV.
FIGURE 3  Differential expression of collagen types I and IV by the sheath and core tissues of rabbit ACL. A,B. Collagen type I staining with higher percentages of the core tissues positively stained (yellow arrow) and a lower percentage of sheath positively stained for collagen type I (light blue arrows). C,D. Sheath tissues positively stained with collagen type IV (green arrow in C and D). Lower percentages of the core tissues are positively stained with collagen type IV (white arrow in C and D), and lower percentages of sheath cells express collagen type I (light blue arrows in A and B). E,F. The enlarged areas (green boxes in C and D) showing intense collagen type IV staining. The broken yellow line represents the demarcation line between the sheath and core areas. White bars: 500 µm; yellow bars: 200 µm. ACL, anterior cruciate ligament

FIGURE 2  Characterization of rabbit ACL by H&E and immunohistochemical staining. A. The horizontal section of ACL shows two distinct parts, loose sheath (green box) with disorganized collagen fibers (green arrows) and dense core (blue box) with organized collagen fibers (blue arrows) by H&E staining. There are also some blood vessel-like structures in the sheath area (back arrows). B. Enlarged area of sheath (green box in A) clearly shows loose fiber net mesh and vessel-like structures (black arrow). C. Enlarged area of core (blue box in A) shows well-organized dense collagen fibers. D. Immunostaining of cross-section with CD105 shows sheath area with intense staining (green box) while core part hardly shows any staining (blue box). E. Enlarged area of the sheath tissue (green box in D) with blood vessel-like structures positively stained for CD105 (white arrows). F. Enlarged area of core tissue (blue box in D) shows few cells positively stained for CD105. G. The sheath area positively stained by CD31 (yellow arrow). H. Enlarged area of sheath tissue (green box in G) with blood vessel-like structures positively stained for CD31 (yellow arrow). I. Enlarged area of core tissue (blue box in G) shows negative staining for CD31. J. Immunostaining of cross-section with CD34 shows the sheath area was positively stained for CD34 (green box). K. Enlarged area (green box in J) of positive CD34 staining. L. Negative staining for CD34 in core. M. The ACL tissue was stained with tenascin C. N. Enlarged area of the sheath tissue (green box in M) was negatively stained for tenascin C. O. Enlarged area of the core tissue (blue box in M) was positively stained for tenascin C. Yellow bars: 200 µm; green bars: 100 µm; and white bars: 50 µm. ACL, anterior cruciate ligament; H&E, hematoxylin and eosin
(basement membrane marker). The results indicated that the sheath tissue contained pericytes as evidenced by CD105 positive staining (Figure 2D [green box] and 2E) compared with the core tissue (Figure 2D [blue box] and 2F). In addition, CD31 was highly expressed in the sheath, but not in core (Figure 2G-I). The expression of CD34 was also markedly higher in the sheath in comparison to the core which did not express any CD34 (Figure 2F). Furthermore, higher percentages of the core tissues were positively stained by tenascin C than that of the sheath tissues (Figure 2M-O). Moreover, stronger expression of col I was found in the core tissue (Figure 3A and 3B), but much less staining was present in the sheath area (Figure 3A and 3B). However, the sheath tissues were positively stained with col IV, with much less staining in the core (Figure 3C-F).

3.2 | Differential colony forming potential of ACL-S and ACL-C SCs

Primary culture showed that both ACL-S and ACL-C SCs formed circular colonies at day 10 (Figure 4A and 4C). However, the colony numbers and the size were different. Higher numbers and larger colonies were formed by the ACL-S SCs (Figure 4B, 4E, and 4F) compared with the ACL-C SCs (Figure 4D-F) at day 15. Specifically, when the cells were grown in a 60-mm-diameter dish, ACL-S SCs formed 1.6 times more colonies than ACL-C cells (Figure 4E). The average colony size of ACL-S cells was twice that of ACL-C cells (Figure 4F).

3.3 | Differential cell proliferation potential of ACL-S and ACL-C SCs

The above experimental results indicated that the ACL-S cells grew much quicker than ACL-C cells during primary culture. Both types of cells at passage 2 still maintained cobble-stone-like cell shape (Figure 5A and 5B). However, the density of ACL-S cells was much higher than that of ACL-C cells. Proliferation assay results demonstrated that the ACL-S cells proliferated 1.7 times faster than the ACL-C cells at days 1 and 3 (Figure 5C).

3.4 | Differential SC marker and collagen expression in ACL-S and ACL-C SCs

Immunocytochemistry staining of these cells showed that both sheath and core cells in culture at passages 2 still expressed SC markers Oct-4 (Figure 6A and 6B) and NS (Figure 6C and 6D), which were located in the cell nucleus. Similarly, human ACL-derived SCs express Oct-4, NS, and SSEA-4.8 Semi-quantification showed that approximately 78% of ACL-S cells were positive for Oct-4 compared with 62% for ACL-C cells, and the difference between the two was significant. No significant difference was found on the percentage of NS positive cells between the ACL-S and ACL-C group (Figure 6E). Furthermore, 96% of ACL-C cells were positively stained for col I (Figure 7B and 7E); however, only approximately 52% of ACL-S cells were positively stained for col I (Figure 7A and 7E). On the other hand, 81% of ACL-S cells were positively stained for col IV (Figure 7C and 7E), while only 11% of ACL-C cells expressed col IV (Figure 7D and 7E).

3.5 | Different extent of multidifferentiation in ACL-S and ACL-C SCs

The multidifferentiation potential of ACL-S and ACL-C cells was tested by culturing the cells with three differentiation induction media (adipogenic, chondrogenic, osteogenic) for 14 days. The chemical staining results showed that both ACL-S and ACL-C cells possessed multidifferentiation potential. However, ACL-S cells had

**FIGURE 4** Differential capacity in the formation of colonies by ACL sheath (ACL-S) and core (ACL-C) stem cells in primary culture. A, The colonies of ACL-S cells. B, A typical colony of ACL-S cells 15 days in culture. C, The colonies of ACL-C cells. D, A typical colony of ACL-C cells 15 days in culture. E, Total colony number is significantly greater in ACL-S cells compared with ACL-C cells. F, The average colony size of ACL-S is also significantly larger than ACL-C cells. Bars: 100 µm. Figures are representatives from three different experiments using cells isolated from three ACL tissues. The colony number and size were determined in each dish (n = 10). ACL, anterior cruciate ligament
a higher degree of adipogenic potential than ACL-C cells (Figure 8). Many obvious intracellular lipid vacuoles positively stained (more than 75%) by Oil Red O were found in ACL-S cells (Figure 8A); however, only 30% of ACL-C cells were positively stained (Figure 8B). Semi-quantification indicated that the lipid staining in the ACL-S cell group was about 1.5 times more than that of the ACL-C cell group (Figure 8C).

In addition, when both cell populations were cultured in chondrogenic medium for 14 days, the chondrogenic-differentiated cells showed a marked production of glycosaminoglycans (GAGs), which was intensely stained by Safranin O dye (Figure 9). ACL-S cells that had undergone chondrogenic differentiation produced significantly greater GAG deposition (Figure 9A and 9C) compared with that of such differentiated ACL-C cells (Figure 9B and 9C).

Furthermore, after 14 days of culture in osteogenic medium, the calcium deposition, as revealed by Alizarin Red S staining and extraction, was significantly higher in ACL-S (Figure 10A and 10C) in comparison with ACL-C cells (Figure 10B and 10C).

4 | DISCUSSION

This study shows that the ACL of the rabbit has a distinct anatomy as one bundle, without a well-defined septum in contrast to human ACL. Also, it comprises two distinct types of tissue components, namely, sheath and core, which display differential biological properties in terms of organization, collagen type, and presence of vessels. Moreover, the cells in the sheath and the core of ACL exhibited SC/progenitor characteristics. The sheath cells were able to form a higher number and larger colonies and proliferated more rapidly than the cells from the core of the ligament. Analysis of multiple differentiation indicated a greater differentiation potential for cells from the sheath tissue compared to cells from the core tissue of ACL.

It is known that the extracellular matrix plays an important role in providing mechanical support, guiding tissue in growth, and maintaining homeostasis during the ligament regeneration process. It has been reported that ACL contains a large quantity of col I, which is about 95%
of the total ligamentous collagen, and small amounts of col III, col IV, col V, and col XII. Our results showed that ACL-C tissue contains a large amount of col I and small amount of tenascin C, indicating that the core tissue is ligament-like tissue.

In this study, we showed that CD105, CD31, and CD34 (three vascular cell markers), and col IV (basement membrane marker) were located in great amounts in the sheath region of the ACL. This is in contrast with the core region that was stained with minimal CD105, CD31, and CD34, and lower levels of col IV.

Thus, the sheath is richly vascularized with basement membrane and hence the sheath cells identified in this study are vascular in origin. On the other hand, the core tissue is of a ligamentous origin, and the core cells in the tissue are connective tissue cells. The vascularized sheath tissue, because of its abundant blood vessels, contains more SCs than core tissue. The core tissue is relatively avascular connective tissue like tendon, which contains fewer cells in general and SCs in particular. Moreover, the sheath cells have better stemness than the core cells (Figures 6 and 8), which are more like ligamentous progenitor cells.

Past studies have demonstrated that human ACL tissue contains populations of cells that share the characteristics of MSCs in terms of differentiation markers or multipotency. It should be noted that most of the previous basic studies have considered ACL as a single unit. Hence, in light of the findings of this study, it seems clear now that MSCs in these studies were a mix of vascular SCs from the sheath and ligamentous SCs from the core of ACL. Previously, we showed that human ACL SCs and medial collateral ligament (MCL)
SCs exhibit differential properties in terms of clonogenicity, self-renewal, and multidifferentiation potential. These findings indicate that the biological properties of ligament stem/progenitor cells are not only dependent on the anatomic location of the “component tissue” in the ligament, as shown in this study, they also vary among different types of ligaments like ACL and MCL. Indeed, a recent comparative study of human MSCs from bone marrow and adipose tissue SCs suggested that tissue source determines the differentiation potential of MSCs possibly due to the differential methylation status of the transcription factors controlling MSC fate.

Currently, the “gold standard” in the clinical routine to treat ACL tears is surgical ACL reconstruction using autologous graft. As for the poor intrinsic regenerative properties of ACL, several critical issues, such as reinjury, delayed biological incorporation, and cartilage degeneration, occur postoperatively. Therefore, many biological strategies, including active substances like platelet-rich plasma and growth factors, gene transfer, tissue engineering, and SC therapy have been introduced and aggressively developed in recent years for better healing and repair of injured ACL.

In this study, we have isolated two distinct tissues, sheath and core from ACL and characterized their respective SCs individually in contrast to the isolation of mixed populations as having been done in previous studies. The sheath is of vascular origin, whereas core is of ligamentous origin and as a result, sheath SCs differ greatly from core SCs in their biological properties. Both types of SCs participate in repair of injured ACL. Therefore, to restore the normal structure to injured ACL in future treatment, both types of SC may have

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**FIGURE 8** The differential extent of adipogenic differentiation by ACL-S and ACL-C stem cells. Oil red O staining for adipogenesis shows ACL-S stem cells with more extensive staining compared to ACL-C cells (A,B). Inset box in each image shows enlarged positively stained lipid drops. The quantification shows significantly higher amounts of lipids in ACL-S cells compared with ACL-C cells (C) (*P < 0.05). Bars: 100 µm. ACL-C, anterior cruciate ligament core; ACL-S, anterior cruciate ligament sheath; OD, optical density.

**FIGURE 9** The differential extent of chondrogenic differentiation by ACL-S and ACL-C stem cells. Safranin O staining for chondrogenesis shows ACL-S cells with increased staining compared to ACL-C cells (A,B). The quantification shows ACL-S cells with significantly higher staining for GAGs compared with ACL-C cells (C) (*P < 0.05). Bars: 100 µm. ACL-C, anterior cruciate ligament core; ACL-S, anterior cruciate ligament sheath; GAG, glycosaminoglycan; OD, optical density.

**FIGURE 10** The differential extent of osteogenic differentiation by ACL-S and ACL-C stem cells. Alizarin Red S staining for osteogenesis shows more extensive staining in ACL-S cells compared to ACL-C cells (A,B). The quantification was done by three wells of each group and the results confirmed the finding that calcium deposition on ACL-S cells is significantly greater than ACL-C cells (C) (*P < 0.05). Bars: 100 µm. ACL-C, anterior cruciate ligament core; ACL-S, anterior cruciate ligament sheath; OD, optical density.
to be used to form sheath tissue by ACL-S and core tissue by
ACL-C cells. On the other hand, sheath SCs proliferate faster
and maintain better “stemness” than core SCs, hence sheath
SCs are expected to promote ACL injury healing and repair
more effectively than core SCs.

Two issues related to this study should be discussed.
The first is the potential “purity” of the SCs isolated from
this study. As we discussed above, the sheath SCs are of
vascular nature whereas the core SCs are of ligamentous
origin (see Figure 7). Furthermore, we showed that these
two types of SCs differ in the number and size of colonies,
the rate of their proliferation, and the extent of their
multidifferentiation. Also, previous studies have shown
that the use of single cell suspension, as used in this
study, produces relatively “pure” SCs,\(^{10,20}\) because other
types of cells, mostly fibroblasts, do not form colonies;
rather, they spread around. However, it is true that
because of the small size of tissue samples, it is difficult to
separate the sheath completely from the core of ACL.
Therefore, we believe that the core SC culture also
contained some sheath SCs. This may explain why low
levels of col IV, which is a vascular tissue marker, were
expressed in the core SCs (Figure 7D).

The second issue is about using the terms of sheath
and core SCs. It is well established that MSCs are adult
SCs located in nearly all tissues and organs in body.
Hence, there is little doubt that these sheath and core
SCs are MSCs, although they are specific to ACL-S and
core tissues. Indeed, previous studies that isolated SCs
from whole human ACL called them MSCs.\(^{6,7}\) These
ACL-derived MSCs express a panel of markers, including
CD105, CD166, CD73, CD90, CD44, CD29, and CD13.\(^{14}\)
However, strictly speaking, once SCs, like ACL-S and
ACL-C cells in study and indeed MSCs, are removed from
their native environment and grown in culture, they are
not “authentic” SCs per se; rather, they are progenitor
cells. So, the term “stem cell” in general is only nominal.

5 | CONCLUSION

We showed in this study that the rabbit ACL comprises two
distinct types of tissues, the sheath and core. The sheath
and core tissues contain vascular and ligamentous SCs,
respectively, that exhibit different biological properties.
These SCs may play a differential role in ligament healing
and regeneration. They may serve as two promising cell
sources for effective repair of injured ACLs.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

BC performed experiments, evaluated the data, and drafted
the manuscript. GZ and DN helped perform the experiments
and revised the manuscript. FF participated in the discus-
sion of this study and supervised the project. JZ performed
experiments, analyzed the data, and drafted the manuscript.
JHW conceived the study, provided feedback in the study
design and data analysis, and revised the manuscript.

ETHICAL APPROVAL

The experimental procedures were conducted in accor-
dance with ethical standards and according to the
Declaration of Helsinki and according to National and
International guidelines. The protocol for use of the rabbits
was approved by the IACUC of the University of Pittsburgh.

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