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Tendon contains more stem cells than bone at the rotator cuff repair site

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Background: The rotator cuff (RC) repair failure rate is high. Tendon and bone represent sources of mesenchymal stem cells (MSCs), but the number of MSCs from each has not been compared. Bone channeling may increase bone-derived MSC numbers participating in enthesis re-formation at the "footprint" repair site. The effect of preoperative channeling on increasing bone MSC numbers has never been reported. We asked (1) whether bone contains more MSCs than tendon at the time of arthroscopic repair and (2) whether bone preoperative channeling at the RC repair site increases the number of bone-derived MSCs at the time of surgery.
Methods: In 23 participants undergoing arthroscopic RC repair, bone was sampled from the footprint and tendon was sampled from the distal supraspinatus. We randomized participants to the channeling or no-channeling group 5 to 7 days before surgery. We enumerated MSCs from both tissues using the colony-forming unit–fibroblast (CFU-F) assay (10 per group). We identified MSC identity using flow cytometry and MSC tri-differentiation capacity (n = 3).
Results: Tendon CFU-F per gram exceeded bone CFU-F per gram for both groups (479 ± 173 CFU-F/g vs. 162 ± 54 CFU-F/g for channeling [$P = .036$] and 1334 ± 393 CFU-F/g vs. 284 ± 88 CFU-F/g for no channeling [$P = .009$]). Ninety-nine percent of cultured cells satisfied the MSC definition criteria.
Conclusions: The distal supraspinatus tendon contained more MSCs per gram than the humeral footprint. Tendon may represent an important and overlooked MSC source for postoperative enthesis re-formation. Further studies are needed to evaluate the repair role of tendon MSCs and to recommend bone channeling in RC repair.
Level of evidence: Basic Science Study; Histology

Keywords: Mesenchymal stem cells; rotator cuff; arthroscopy; enthesis; bone channeling; tendon; anchor repair

All protocols described in this study were approved by the Ottawa Health Sciences Network Research Ethics Board (REB No. 2009042-01H).

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Rotator cuff (RC) tears impair function and represent the most common cause of shoulder pain in humans.[28,44] Nearly one-third of individuals older than 60 years and up to half of those aged 80 years or older have RC tears, representing a significant health burden.[22,32] Torn RC tendons can be surgically repaired; however, repairs fail to heal at rates ranging from 11% to 40%, with a rate of over 90% for massive tears despite newer surgical techniques.[3,11,21,29,32,33,43]

Mesenchymal stem cells (MSCs) have been identified in most tissues throughout the body.[42] Autologous bone marrow–derived cells, including MSCs, participate in tendon-to-bone biological integration and enthesis re-formation after RC surgical repair.[6,17,19] Bone marrow–derived MSCs may play a major role in RC healing owing to their immediate proximity to the surgical site, as well as their ability to proliferate and differentiate into the necessary cell types to form a new enthesis.[16,18,19] Although less frequently performed today, open surgical repair uses transosseous tunnels through which sutures are passed to the lateral aspect of the greater tuberosity.[25] This approach ensures close contact of the deep marrow with the repaired tendon.[39] The modern-day arthroscopic approach has resulted in a change in technique: Anchors now hold the tendon over the insertion area (footprint) without direct access to the bone marrow.[36] To augment access to the marrow and its pluripotent cells, many shoulder surgeons decorticate the footprint bone plate at the time of surgery to a depth of 1 to 2 mm to open communication between the tendon stump and the superficial, subentheseal bone marrow.[14,24] In older adults, however, superficial bone marrow contains mainly fatty marrow and decortication may fail to access the deep red marrow.[27] Should the deep bone marrow be the primary source of the MSCs necessary for RC regeneration, arthroscopic anchor repair could delay the re-formation of the enthesis and adversely affect postsurgical outcomes.[27] As a further augmentative step, shoulder surgeons have performed channeling, described as drilling the subentheseal bone to more than 1 cm deep, to open communication between the site of repair and the deeper red bone marrow.[14,24] Arthroscopic surgical outcomes are comparable with those of open repair.[4,23,26] Although this might suggest that MSCs are not required for postsurgical healing, it should also be considered that another important source of MSCs is available: the distal supraspinatus (SSP) tendon.

The primary purpose of this study was to compare the number of MSCs present in the SSP tendon and bone at the site of RC repair at the time of arthroscopic surgery. The secondary purpose was to determine whether preoperative channeling increased the number of MSCs present in bone at the time of surgery. Our first hypothesis was that the number of bone-derived MSCs would exceed that of the distal SSP. Our second hypothesis was that we could potentiate the number of bone marrow–derived MSCs at the footprint on the day of surgical repair by performing preoperative channeling 5 to 7 days before arthroscopy.

Materials and methods

Patients and channeling protocol

This study is an exploratory basic science investigation that was a late addition to a registered clinical trial listed at ClinicalTrials.gov (identifier NCT01877772). Because we developed the outcomes from this basic science study late into study recruitment, they are not listed in the study protocol and thus did not alter the registration. Other outcomes described in the registration protocol will be reported in a future publication. We recruited patients aged 18 years or older with full-thickness SSP tears in whom 6 months of conservative treatment had failed between August 2014 and March 2016. The exclusion criteria included acute tears; characteristics of the cuff tear that rendered the cuff irreparable (fatty infiltration in the muscles of grade III [50%] or greater, superior subluxation of the humeral head, or retraction of the cuff to the level of the glenoid rim); previous surgery on the affected shoulder; active joint or systemic infection; significant muscle paralysis, and RC tear arthropathy (ClinicalTrials.gov shows a complete list). All the individuals participating in the study provided their written consent. All personnel (including the surgeon), with the exception of the interventional radiologist performing the intervention, and all patients were blinded to group allocation until all data had been collected and analyzed. Five to seven days prior to RC repair, we allocated patients to 1 of 2 groups using a block randomization allocation sequence: channeling of the proximal humerus at the site of surgical repair (footprint) or no-channeling treatment. We chose this time frame based on histologic data from a rabbit model showing that more bone-lining cells were present 5 to 7 days after preoperative channeling (Supplementary Figure S1).

Channeling in our participants consisted of ultrasound assessment of the RC with a 17-MHz ultrasound probe; a standard chlorhexidine skin preparation and draping; and infiltration of 10 mL of local 2% lidocaine subcutaneously, in the subacromial space and at the footprint. Under sterile technique and by use of ultrasound guidance, a 10-cm-long, 2-mm-diameter K-wire was advanced onto the RC footprint percutaneously to within 5 mm of the proximal end of the K-wire. We drilled 4 holes along the anteromedial, posteromedial, anterolateral, and posterolateral margins of the footprint in a standardized ‘‘box’’ pattern to a depth of 9.5 cm into the humeral metaphysis. We chose the wire diameter and number of holes to cover the human SSP footprint area while minimizing the risk of fracture or instability. We selected the depth to reach the red marrow in the proximal metaphysis and diaphysis,[41,45] verifying this on a cadaveric shoulder. Channeling was performed proximal to the surgical anchor repair sites (at the footprint) and therefore did not affect the placement of the anchors. Patients allocated to the no-channeling group underwent a sham operation that consisted of the same procedure: ultrasound evaluation; infiltration of local anesthetic; and percutaneous placement of the K-wire, with activation of the drill at the RC footprint, but without advancement of the drill into bone.

In all cases, immediately prior to arthroscopic RC repair, we harvested approximately 100 mg of bone from the superficial aspect of the cuff footprint with a pituitary rongeur, taking care to

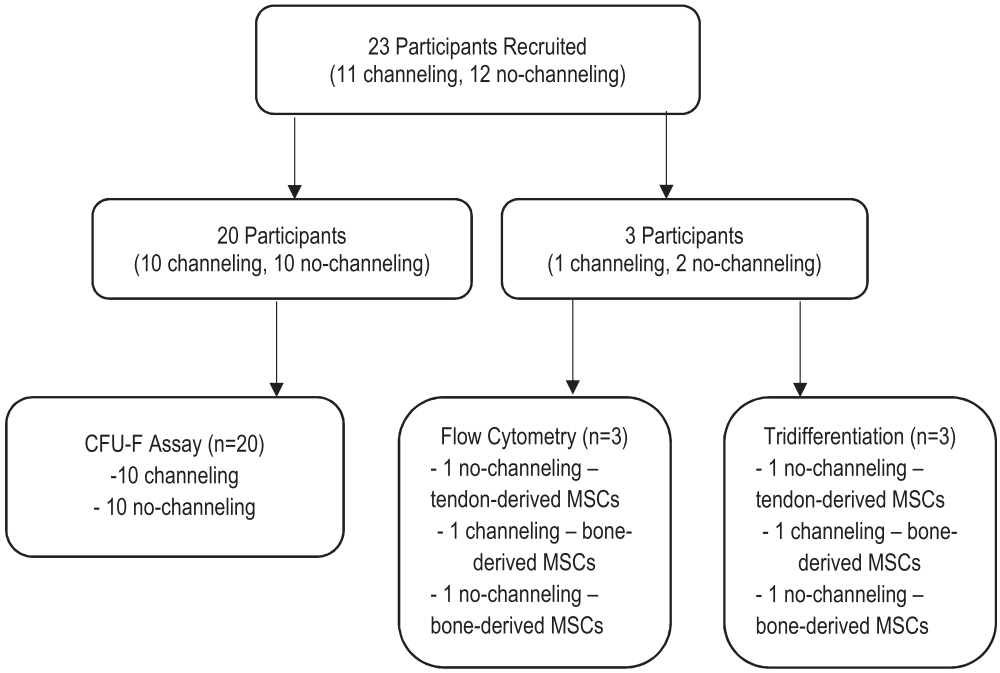

Figure 1 Participant group allocation flow diagram. *CFU-F*, colony-forming unit–fibroblast; *MSCs*, mesenchymal stem cells.

sample all aspects of the RC insertion area. In addition, in all cases, we harvested tissue (approximately 60 mg) from the distal torn tendon edges with a basket forceps. Bone and tendon tissue was completely separated; placed in separate containers, in low-glucose Dulbecco modified Eagle medium with 1% penicillin-streptomycin; and then brought immediately to the laboratory for processing.

Cell isolation

We separately minced tendon and bone samples using a standardized protocol with a scalpel under sterile conditions. Laboratory staff, blinded to allocation, processed the samples for cell isolation and all downstream experiments. We placed minced tissue in StemMACS MSC expansion media (Miltenyi Biotec, Bergisch Gladbach, Germany) containing collagenase at a concentration of 300 units/mL and incubated overnight at 37°C to allow for tissue digest. We washed digested material with phosphate-buffered saline solution and centrifuged at 300*g* for 5 minutes to pellet MSCs and tissue debris. We resuspended cells and tissue debris in a small volume of StemMACS MSC expansion media and plated in a 6-well plate, with 1 well for tendon and 1 for bone. We plated the debris with the liquid fraction to allow MSCs to migrate from the tissue and adhere onto plastic.

Colony-forming unit–fibroblast assay and MSC expansion from collagenase digests

We performed the colony-forming unit–fibroblast (CFU-F) assay as previously described before scoring was performed by an evaluator blinded to both tissue type and group allocation.[5] We divided the number of colonies counted by the number of grams of tissue obtained for the sample to give the number of colonies per gram of tissue. The CFU-F protocol requires that cells be fixed and then stained for counting, preventing cellular expansion and downstream experimentation. We allocated samples from the first 20 participants (10 with channeling and 10 with no channeling) to the CFU-F assay. MSCs from participants providing the remaining samples that were not used for the CFU-F assay were allowed to expand in StemMACS MSC expansion media to passage 3 for flow cytometry and trilineage differentiation experiments (Fig. 1).

Flow cytometry

We analyzed passage 3 primary culture MSCs at the StemCore Cell Sorting Facility (Ottawa, ON, Canada) using a MoFlo XDP flow cytometer (Beckman Coulter, Brea, CA, USA). We resuspended 100,000 cells in 50 μL of fluorescence-activated cell sorting buffer (phosphate-buffered saline solution plus 0.5% bovine serum albumin) and conjugated antibodies. We performed staining for CD90, CD73, CD105, CD45, CD34, CD11b, CD19, and Human Leukocyte Antigen–DR isotype (HLA-DR) using the BD Stemflow Human MSC Analysis Kit (BD Biosciences, San Jose, CA, USA). We included isotype antibodies with the kit as negative controls. We analyzed all flow cytometry data using FlowJo analysis software (FlowJo, Ashland, OR, USA).

Trilineage differentiation

We induced passage 3 MSCs from tendon and bone samples toward osteogenesis, chondrogenesis, and adipogenesis using the human MSC functional identification kit (R&D Systems,

Minneapolis, MN, USA). We allowed cultures to differentiate for 21 days, according to standard protocols. We evaluated differentiation using immunofluorescence for the detection of osteocalcin (osteocytes), aggrecan (chondrocytes), and FABP4 (adipocytes). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). We evaluated relative staining for each fluorophore qualitatively using fluorescence microscopy.

Statistics

We were not aware of any data on the basis of which to perform a power analysis for our primary outcome and first hypothesis. We therefore used data from our rabbit shoulders, from which we estimated that bone would supply approximately 50% more MSCs than tendon with a standard deviation of 40%. Using 2-sided statistical testing with a β of .8 and an α of .5, we estimated that 10 participants per group should show a statistically significant between-group difference. We performed statistical testing using SPSS software (version 16.0; IBM, Armonk, NY, USA). We tested numerical differences between tendon- and bone-derived MSCs, as well as for channeling vs. no channeling, using the Mann-Whitney *U* test for nonparametric data. The Spearman correlation coefficient tested for correlations. We considered differences to be statistically significant at $P \leq .05$.

Results

Participant recruitment

We recruited 23 participants: 11 in the channeling group and 12 in the no-channeling group (Fig. 1). The mean age was 59 ± 11 years (range, 45-79 years) in the channeling group, with 5 of 11 patients (45%) being women, and 58 ± 9 years (range, 45-74 years) in the no-channeling group, with 5 of 12 patients (42%) being women. No adverse events occurred after channeling or no-channeling procedures. A mean of 64 ± 28 mg of tendon tissue and 111 ± 55 mg of bone tissue was harvested and processed to isolate adherent MSC populations from each tissue.

CFU-F counts for tendon vs. bone samples

We initially sought to determine whether stem cell populations could be isolated from harvested tendon and/or bone tissue. The CFU-F colony count was determined for 20 participants (n = 10 from each group) for adherent cells isolated by enzymatic digestion of the tissues. The mean CFU-F colony count per gram of tissue was greater in tendon (479 ± 173 CFU-F/g) than in bone (162 ± 54 CFU-F/g, $P = .036$) for the channeling group and was greater in tendon (1334 ± 393 CFU-F/g) than in bone (284 ± 88 CFU-F/g, $P = .009$) for the no-channeling group (Fig. 2). No correlation was found between age (tendon, $P = .680$; bone, $P = .820$) or sex (tendon, $P > .999$; bone, $P = .431$) and the number of MSCs per gram of tissue.

Effect of channeling on MSC recruitment to footprint

Although the channeling group had lower absolute counts, we found no statistically significant difference in the number of tendon-derived MSC colonies when comparing the channeling shoulders (479 ± 173 CFU-F/g) with the no-channeling shoulders (1334 ± 393 CFU-F/g, $P = .074$). Similarly, MSC colonies per gram of bone in the channeling group (162 ± 54 CFU-F/g) compared with the no-channeling group (284 ± 88 CFU-F/g, $P = .214$) did not reach statistical significance, although absolute counts were lower in the channeling group (Fig. 2).

Both bone- and tendon-derived cells satisfy minimal criteria for MSCs

We next sought to determine the nature of these self-renewing cell populations. We obtained tendon-derived flow cytometry and cell data from 1 no-channeling participant (Fig. 1). We also included bone-derived cells from 1 channeling participant and 1 no-channeling participant. Approximately 99% of live, plastic-adherent cultured cells from all 3 participants were strongly positive for CD73 and CD90 and weakly positive for CD105 (Fig. 3). All CD73-, CD90-, and CD105-positive cells were negative for CD45, CD34, CD11b, CD19, and HLA-DR (Fig. 3). Thus, the self-renewing cell populations isolated from tendon and bone possess cell surface markers consistent with an MSC identity.

To further address the possibility that the tendon- and bone-derived cell populations are MSCs, we performed tri-differentiation assays on these same participant-derived cells (Fig. 1). Immunofluorescence analysis of the plastic-adherent cells from all participants showed that they could be efficiently differentiated into either osteocyte, chondrocyte, or adipocyte lineages (Fig. 4). Thus, the adherent cell populations isolated from tendon and bone possess the characteristics that permit their identification as MSCs.

Discussion

RC disease remains a highly prevalent and disabling condition. The highest rate of early surgical failure occurs from dehiscence at the site of repair.[20] A successful RC repair is one in which, after the initial surgical construct temporarily holds the tendon juxtaposed to the bone, the tissues form a new enthesis that can withstand the required tensile strength.[39] Owing to the absence of pharmacologic treatments to improve enthesis re-formation, regenerative approaches, including the use of MSCs, have become highly attractive.[20] The function of MSCs is niche dependent,

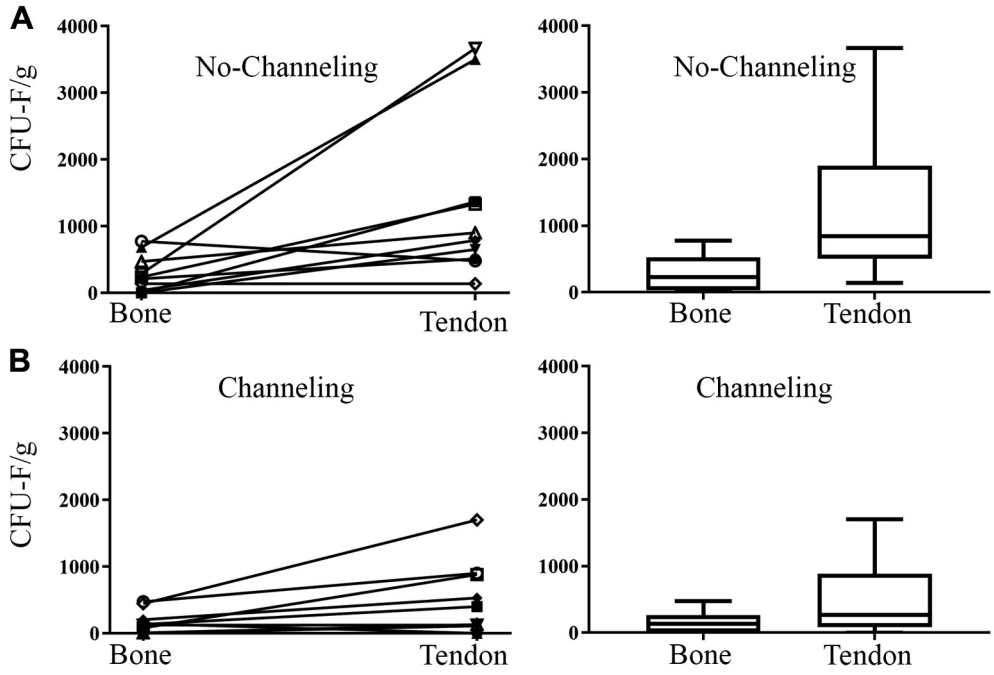

Figure 2 Colony-forming unit–fibroblast (*CFU-F*) count per gram of tissue. (**A**) *Left*, CFU-F per gram per participant in no-channeling group (n = 10), showing that only 1 participant had more bone-derived mesenchymal stem cells (MSCs). *Right*, CFU-F summary data for no-channeling group, showing a higher median number of tendon-derived than bone-derived MSCs. (**B**) *Left*, CFU-F per gram per participant in channeling group (n = 10), showing that only 3 participants had more bone-derived MSCs. *Right*, CFU-F summary data for channeling group, showing a higher median number of tendon-derived than bone-derived MSCs.

indicating that the tissue from which MSCs originate plays an important role in their ability to proliferate and differentiate into various mesenchymal tissues.[7,13] Originally isolated from bone marrow, MSCs have since been found in most tissues.[1] In the past decade, tendon has been identified as a rich source of progenitor cells.[2,31,35,37,40] Bi et al[2] first identified tendons as a source of progenitor cells able to differentiate into mesenchymal lineages and form enthesis-like tissues. Tan et al[35] identified rat patellar tendon MSCs with higher clonogenicity and proliferative capacity; greater tenogenic, osteogenic, chondrogenic, and adipogenic gene expression; and better differentiation potential than marrow-derived MSCs from the femur and tibia. Bone, enthesis, tendon, bursa, and muscle have been identified as sources of MSCs within the shoulder.[24,31,37,40]

We found that tendon samples yielded a mean of 3- to 4-fold more MSCs per gram of tissue than bone. This finding was unexpected given the large cellular reactions in the subentheseal bone and bursa compared with the distal tendon in animal models of RC repair.[38,39] To our knowledge, this is the first study in humans to directly compare the MSC yield between tendon and bone from the same individual, and it identified a greater number of MSCs in tendon compared with bone. Similarly to MSCs, fibroblasts have the ability to adhere to plastic, so we considered whether our tendon-derived CFU-F cell colonies may have represented fibroblasts rather than MSCs. Flow cytometry confirmed that 99.7% showed the same minimal-criteria MSC epitope profile[8] as plastic-adherent cells derived from bone (Fig. 3). Tendon-derived plastic-adherent cells also showed the ability to differentiate into 3 mesenchymal lineages (Fig. 4), similarly to their bone-derived counterparts. These experiments confirmed the identity of the MSCs isolated from both the tendon and the bone sites at RC repair. Previous reports assumed that the hypocellular reaction at the footprint disqualified tendon-derived cells as important contributors to enthesis re-formation.[19,38] Koike et al[19] found a remarkable proliferation of non-chondrocytes after transosseous repair. Referring to data from Uhthoff et al,[38] Koike et al proposed that this cellular proliferation most likely originated in the bone marrow or the bursa, but experiments to confirm the prevalence of MSCs in transosseous repair remain to be performed.[15,34,38,39] Furthermore, MSCs generally represent only a small proportion of cells within any tissue,[12] such that overall cellularity in one tissue might not reliably predict higher numbers of MSCs. In support of our results, Dyment et al[9] identified the paratenon and tendon midsubstance as the main source of stem cells for repair after patellar tendon injury in a mouse model. Ni et al[30] showed that exogenous application of tendon-derived stem cells to a patellar tendon defect in the rat improved both histologic and biomechanical outcomes.

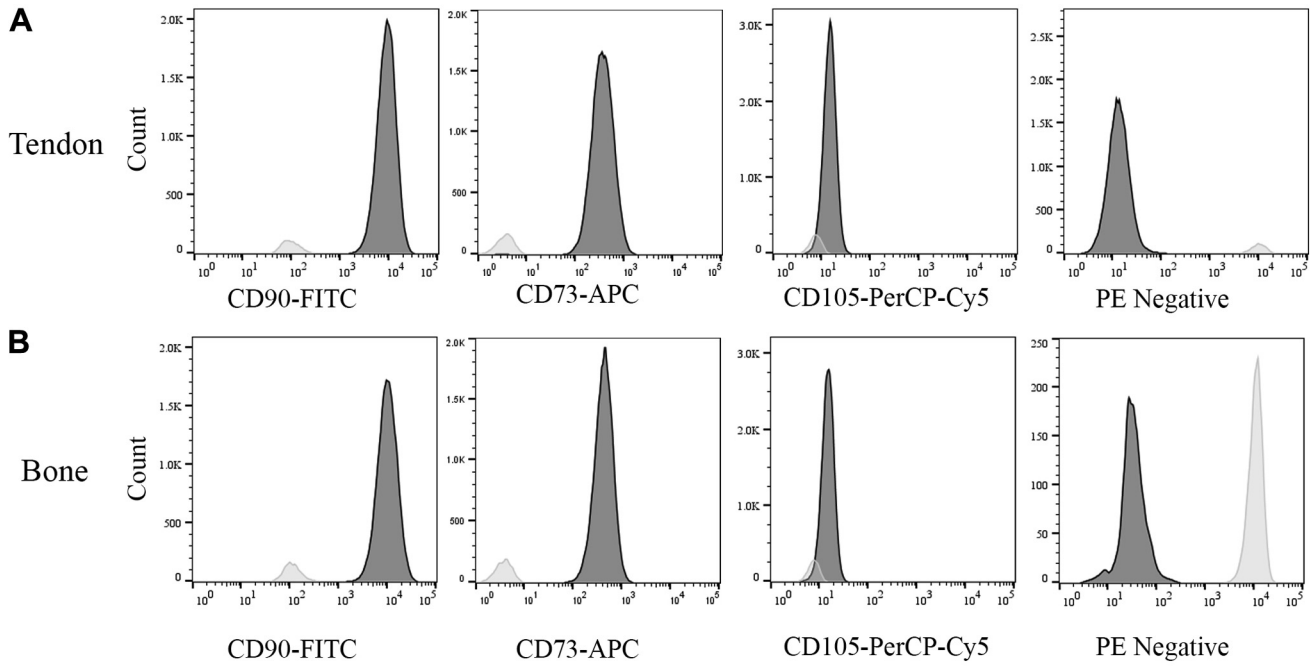

Figure 3 Representative flow cytometry data for bone- and tendon-derived mesenchymal stem cells (MSCs). (**A**) Tendon-derived plastic-adherent cells were positive for CD90, CD73, and CD105 and negative for CD45, CD34, CD11b, CD19, and HLA-DR, consistent with MSCs. (**B**) Bone-derived plastic-adherent cells were positive for CD90, CD73, and CD105 and negative for CD45, CD34, CD11b, CD19, and HLA-DR, consistent with MSCs and similar to tendon-derived MSCs in **A**. *Dark peaks* represent MSC samples, and *light peaks* represent corresponding controls. *FITC*, Fluorescein; *APC*, Allophycocyanin; *PerCP-Cy5*, Peridinin-Chlorophyll-protein-Cy5; *PE*, Phycoerythrin.

This work and the current results support that tendon-derived MSCs may play a larger role in RC enthesis re-formation than previously appreciated. Meticulous surgical attachment of the distal tendon to the footprint may therefore benefit enthesis re-formation, and surgical preparation of the tendon site may be as important as, or more important than, attention devoted to preparing the bony side. Tendon preparation could be performed with simple débridement of the tendon edges with a shaver, piercing the distal tendon in multiple places with a needle or passing instrument or using other techniques that optimize tendon surface contact with the footprint. The potential effect of adding growth factors and chemokines to optimize tendon MSC function and recruitment at the time of surgery requires further exploration. Our participants had been experiencing symptoms for greater than 6 months, and therefore, findings in tendons with acute RC tears may differ; however, previous work using a rabbit model showed no difference in post-repair histologic outcomes after a 12-week delay in tendon repair,[18] and we are not aware of any study that has compared MSCs in tendon after acute vs. chronic tears.

Although clinical outcome measures have not improved after bone channeling at the time of surgery, this intervention increased the integrity of RC repair as visualized on computed tomography and magnetic resonance imaging and decreased the retear rate at 6 and 9 months after repair.[14,15] The rationale for performing preoperative channeling in this study was that accessing the humeral deep bone marrow prior to surgery would increase the recruitment of endogenous MSCs at the time of surgery.[14,15] Although preoperative channeling adds an intervention, the potential benefit of improving surgical outcomes would likely outweigh the inconvenience of the procedure. Although we did not find an increase in the number of MSCs isolated from the footprint at the time of arthroscopic repair or promotion of bone MSCs to surpass non-channeled bone or tendon MSCs in the numbers after channeling, this study was not powered to determine this conclusively. Further study with a larger number of participants is needed. The non–statistically significant difference in mean tendon CFU-F counts between the channeling and no-channeling groups may be attributed to heterogeneity in the number of MSCs per participant, which has been described for both tendon[40] and bone,[5] as well as our relatively small sample size. Additional biological reasons that might account for the lack of recruitment of bone MSCs at the footprint after channeling include the following: (1) If channeling-recruited MSCs had already begun to differentiate toward chondrocytes, their ability to adhere to plastic in vitro could have been compromised and hence they would not be counted using CFU-F assessment. (2) MSC recruitment in part depends on

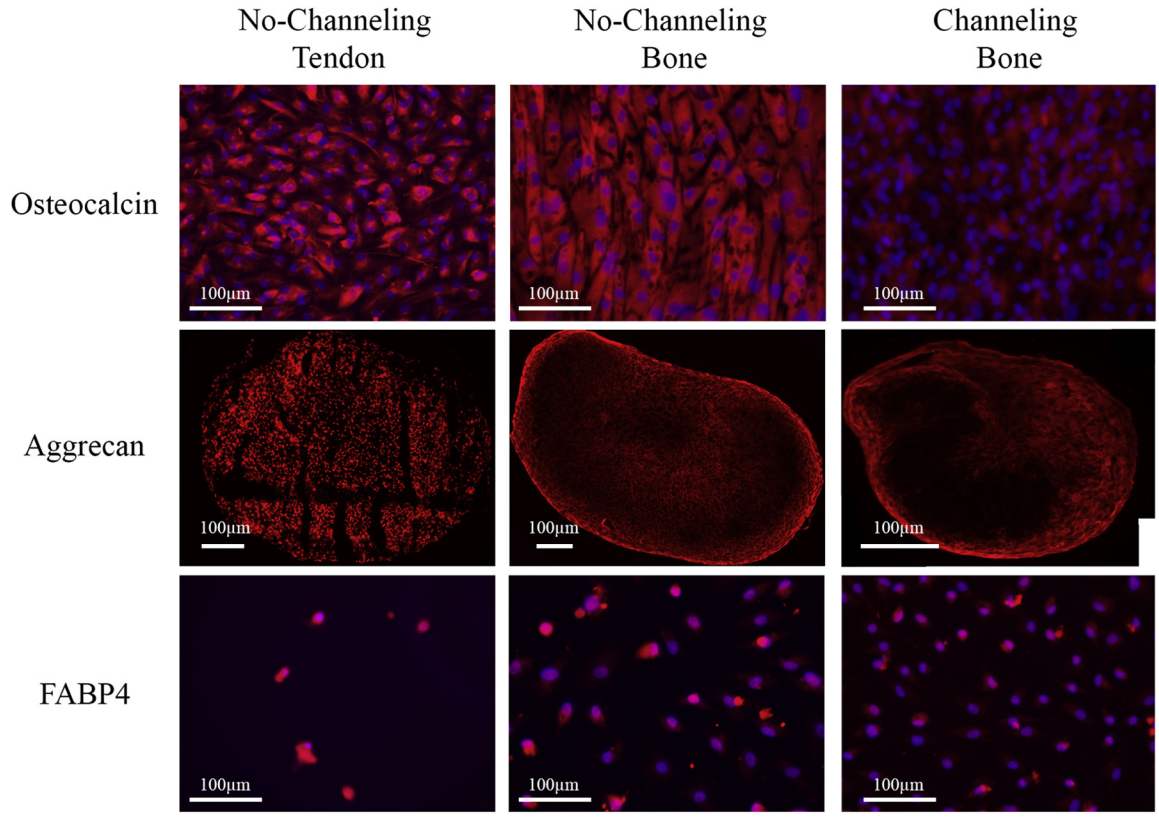

Figure 4 Osteogenic, chondrogenic, and adipogenic differentiation assays for culture-expanded bone- and tendon-derived mesenchymal stem cells. The *top row* presents immunofluorescence (IF) for osteocalcin, showing positive osteogenic differentiation for all expanded samples. The *middle row* presents IF for aggrecan, showing positive chondrogenic differentiation for all expanded samples. The *bottom row* presents IF for FABP4, showing positive adipogenic differentiation for all expanded samples.

micromotion between fragments.[10] In RC repair, channeling did not disrupt the stability of the humerus, and lack of micromotion may have dampened MSC recruitment. (3) The timing of the channeling intervention in this original trial differed from the intraoperative strategy used by other investigators.

Our study has limitations. Although MSC number comparisons between tendon and bone (our primary outcome measure) benefitted from statistical power, post hoc analysis indicated an insufficient sample size to detect a difference between the channeling and no-channeling groups (our secondary outcome measure). This lack of power was a result of the present study being a late addition to an existing study. The 5- to 7-day time frame for preoperative channeling was based on a rabbit model, which may not replicate the shoulder biology of humans. Although we used tissue mass as our denominator for CFU-F comparison, bone and tendon do not have the same density, which could affect the results; however, when we multiplied the bone CFU-F counts by 2 (roughly the density difference between bone and tendon), a statistically significant greater colony count in tendon was still found (mean 2-fold difference, $P = .044$; data not shown). Because we did not include participants with massive RC tears, it is unknown whether such tears may have influenced our CFU-F counts. Finally, we carried out functional evaluation of tendon-derived and bone-derived MSCs or bone channeling and no channeling on 3 expanded MSC cultures solely as confirmatory steps of differentiation capacity, and we did not quantitatively compare the differentiation capacity of each group.

Conclusion

The distal SSP tendon contained more MSCs per gram than humeral bone at the footprint and may therefore represent an important MSC source for postoperative enthesis re-formation. Footprint channeling 5 to 7 days prior to arthroscopic repair did not increase bone MSC numbers at the footprint at the time of repair. This result should be confirmed in a larger study if bone channeling in RC repair is recommended. Our findings may help explain why previous trials failed to find improved clinical outcomes after bone channeling. Further studies

evaluating the role of tendon MSCs in RC repair are needed.

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Supplementary data

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