Rapid Isolation of Human Stem Cells (Connective Tissue Progenitor Cells) From the Proximal Humerus During Arthroscopic Rotator Cuff Surgery

Augustus D. Mazzocca,* MS, MD, Mary Beth R. McCarthy, BS, David M. Chowaniec, BS, Mark P. Cote, PT, DPT, Robert A. Arciero, MD, and Hicham Drissi, PhD
From the University of Connecticut Health Center, Department of Orthopaedic Surgery, MARB Building, Farmington, Connecticut

Background: Bone-to-tendon healing in the shoulder can be unpredictable. Biologic augmentation, through the implementation of adult mesenchymal stem cells, may improve this healing process.

Purpose: The purpose of this study was to (1) arthroscopically obtain bone marrow aspirates from the proximal humerus during rotator cuff repair, (2) purify and concentrate the connective tissue progenitor cells (CTPs) in the operating room efficiently, and (3) confirm these are stem cells through their ability to differentiate into bone cells. We hypothesize that CTPs can be quickly and efficiently isolated from bone marrow during arthroscopic surgery and that these cells are capable of osteogenesis.

Study Design: Cohort study; Level of evidence, 3; and Descriptive laboratory study.

Methods: Bone marrow aspirates were harvested through the anchor tunnel of the humeral head during arthroscopic rotator cuff repair in 23 patients. Twenty-three matched controls were selected from a clinical registry to evaluate for increased incidence of complication. Connective tissue progenitor cells were isolated using 2 accepted methods and compared with a novel, rapid method designed for use in the operating room. Osteogenic potential was assessed by cytochemical and molecular analysis.

Results: Reverse transcription polymerase chain reaction analysis and cellular staining confirmed the osteogenic potential of these CTPs. There was no statistical significant difference in the Single Assessment Numeric Evaluation score (aspirate, 86.3 ± 10.5; control, 83.6 ± 15.1; $P = .54$), range of motion measures (postoperative external rotation: aspirate, 65.0° ± 20.4°; control, 62.5° ± 17.1°; $P = .67$; postoperative forward elevation: aspirate, 163.0° ± 30.6°; control, 145.7° ± 41.4°; $P = .12$), or postoperative strength measures between groups (median, 5; range, 4-5 in the aspirate group compared with median, 5; range, 4-5 in the control group; $P > .05$).

Conclusion: Connective tissue progenitor cells can be safely and efficiently aspirated from the proximal humerus using the anchor tunnel created during arthroscopic rotator cuff surgery. These cells may play an important role in cell-based therapies involving rotator cuff repair.

Clinical Relevance: We have established a reliable, reproducible protocol for isolating CTPs in the operating room. These cells may have the potential to enhance the healing process after rotator cuff repair.

Keywords: stem cells; shoulder; rotator cuff; arthroscopy

Rotator cuff tears are one of the most common causes of pain and disability in the upper extremity and often require operative intervention. Although surgery is widely considered the standard of care, the failure rate after repair of large and massive rotator cuff tears reported in the literature is variable, ranging from 38% to 94%.[15,18,27,36] Studies have shown that bone may play an integral part in the tendon-to-bone reinsertion.[14,18,45,46] Advances have been made to maximize the ability to mechanically repair the rotator cuff, but revision surgery often reveals that the tendon has retracted away from the bone with the suture anchor, corresponding suture material, and knot in place, raising suspicion of a biologic healing problem.[7,13,31,52]

*Address correspondence to Augustus D. Mazzocca, MS, MD, University of Connecticut, Department of Orthopaedic Surgery, Farmington, CT 06034 (e-mail: mazzocca@uchc.edu).

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Bone marrow (BM) is a viable source of stem cells, commonly called connective tissue progenitor cells (CTPs), which can be differentiated into bone, tendon, cartilage, and ligament.[5,9,23,32,41,42,47] Connective tissue progenitor cells are characterized by their ability to undergo self-renewal, multilineage differentiation, and form terminally differentiated cells. Bone marrow represents the most commonly used and studied source of CTPs, which may have the potential to aid in the progression of healing tissues because of their pluripotency.

To use CTPs for healing enhancement, an efficient and safe method of obtaining BM during shoulder surgery needs to be developed. Connective tissue progenitor cells have been successfully isolated from the iliac crest and used for bone regeneration therapy.[33] Although this method is well documented, it is not ideal to use the iliac crest as a source of BM during arthroscopic rotator cuff repair (RCR) as it adds an additional procedure and increases patient morbidity. The epiphysis of the proximal humerus is made up of mostly trabecular bone and is thought to be a rich source of hematopoietic cells.[34] The proximal humerus may be a suitable site for aspirating BM as there is adequate exposure of the humeral head and greater tuberosity during arthroscopic RCR surgery, eliminating the need for an additional procedure.

The purposes of this study were to determine (1) if BM could be safely aspirated from the proximal humerus efficiently without an increase in patient morbidity and with no compromise in sterility during arthroscopic RCR, (2) if BM could be quickly and safely purified in the operating room (OR) to isolate a fraction rich in CTPs, and (3) if this fraction contained CTPs capable of osteogenesis, confirming the presence of stem cells. Our hypothesis was that BM can be safely obtained from the proximal humerus at the time of arthroscopic RCR surgery in an efficient manner without any compromise in sterility. Further, this BM can be quickly purified in the OR to isolate a fraction rich in CTPs that are capable of osteogenesis.

MATERIALS AND METHODS

Inclusion and Exclusion Criteria

All patients undergoing arthroscopic RCR from a single surgeon (A.D.M.) were considered eligible for this study (institutional review board No. 06-577-1). Exclusion criteria included patients who were pregnant, prisoners, and those with a history of diabetes, hepatitis, human immunodeficiency virus, or AIDS, or patients who had been subjected to radiation. All recruited patients were mature individuals, 41 to 76 years of age, who had elected to undergo arthroscopic RCR. The patients read and signed a consent form before enrollment.

Clinical Outcome and Morbidity

Ninety arthroscopic RCRs were performed in 89 patients by a single surgeon (A.D.M.) from September 2006 through June 2008. Of these 89 patients, 23 consented to the harvesting of BM as part of an investigative study examining various characteristics of CTPs from human BM. From the remaining 66 patients who underwent arthroscopic RCR, 23 matched controls were selected to evaluate for increased incidence of complication (institutional review board No. 08-057-3). These controls were selected from the same surgeon's practice and matched for age, sex, and follow-up time to allow for accurate comparisons. Controls were selected by an independent reviewer who was blinded to all outcome data. The remaining 43 patients (44 repairs) who underwent arthroscopic RCR in this period but who had not undergone aspiration and were not appropriate for selection as controls were also evaluated.

The medical records for this study and controls were reviewed to determine the presence of surgical or postoperative complications. Surgical complications included events or technical difficulties that occurred intraoperatively that were directly related to the harvesting of BM (eg, needle breaking off in humeral head, fracture as a result of needle punch, suture hole enlargement, puncture of the neurovascular structures of the axilla secondary to the needle penetrating through the humeral head). Postoperative complications included infection, reflex sympathetic dystrophy (RSD), deep venous thrombosis (DVT), septic arthritis, stiffness, wound irregularities, hematoma, abnormal pain defined as continued postoperative pain of more than 3 months, and delayed healing or failure of the repair or reconstruction. The Single Assessment Numeric Evaluation (SANE) score, postoperative range of motion (external rotation and forward elevation), and measure of strength were collected and compared to evaluate whether a difference in postoperative outcome existed between these 2 groups.[50]

Forward elevation was measured actively and external rotation was measured passively with the arm at the side. Visual estimation was used to quantify degrees of motion. Visual estimation measurements of shoulder range of motion have demonstrated good intra- and interrater reliability. As stiffness after surgery was a concern, all range of motion measures were assessed bilaterally. Postoperative stiffness was defined as a difference of 20° or greater in forward elevation and or 10° or greater in external rotation, and was graded as either being present or absent. Strength was measured in a position of scaption with manual muscle testing based on the Medical Research Council Scale for Muscle Strength.[34] This measure was collected bilaterally, with the uninvolved side serving as the reference index for strength grading.[19]

Collection of Bone Marrow Aspirate

Bone marrow aspirate (BMA) was collected during arthroscopic RCR. All repairs were performed using PEEK (polyetheretherkeytone) 5.5-mm suture anchors and high-strength, nonabsorable No. 2 suture (Arthrex, Inc, Naples, Florida). Repair type and anchor configuration were determined based on type of tear and quality of the tissue. Before anchor placement, a 14-gauge aspiration

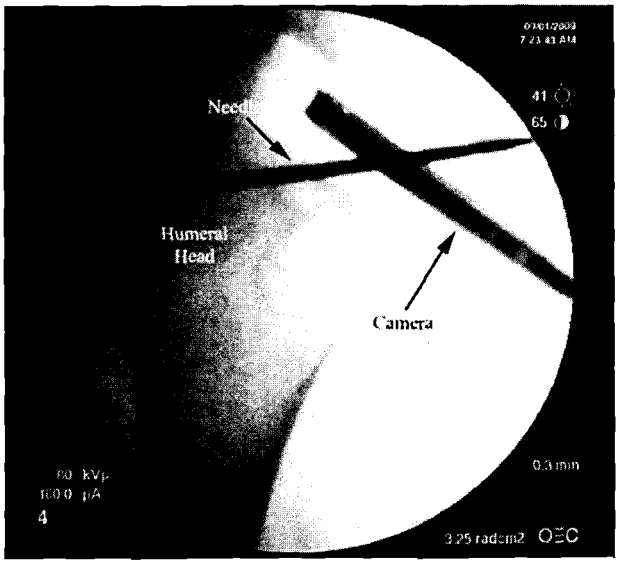

Figure 1. Bone marrow aspiration from the proximal humerus during arthroscopic rotator cuff repair.

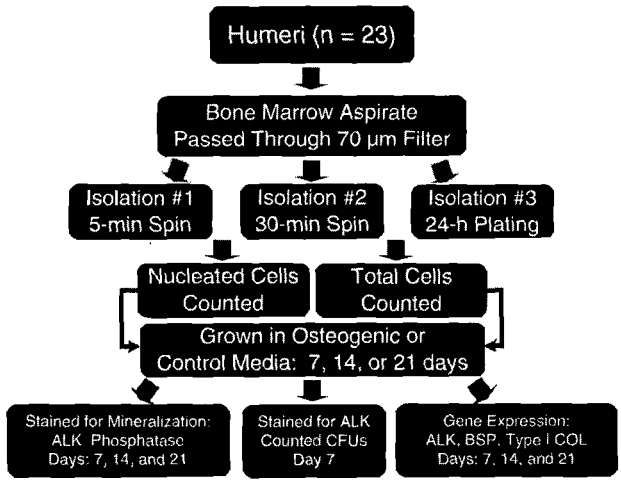

Figure 2. Experimental outline of the isolation protocol.

needle (Arthrex) fitted with a 60-mL syringe containing 1 mL of 1000 U of preservative-free sodium heparin as an anticoagulant in 9 mL of saline was placed 25 mm into the medullary cortex at the bone cartilage junction of the footprint where the first suture anchor would be placed (Figure 1). To standardize the method of aspiration, the surgeon pulled back on the syringe to maximize suction for 60 seconds, allowing BM to flow into the syringe. The needle was removed and the anchor was placed directly into the tunnel created by the needle. The needle gauge was chosen to accommodate the 5.5-mm diameter suture anchor used in the RCR procedures. Aspiration of peripheral blood and air bubbles was minimized by performing the procedure under 40 mm Hg constant fluid pressure maintained by an arthroscopic fluid pump (Arthrex). A surgical technician carefully overlaid the BM onto a 17.5% sucrose gradient in a 50-mL conical tube (Fisher Scientific, Agawam, Massachusetts). The tube was capped, passed off the surgical field to the circulating OR nurse, and centrifuged for 5 minutes at 205 *g*. The fractionated top yellow layer was easily distinguished from the pink middle layer and was drawn up by the surgical technician using the same aspiration needle used to obtain the BM and taken to the laboratory for contamination testing. The process took approximately 10 minutes and did not impair the work flow in the OR. This protocol was reviewed by the university's Office of Research Safety and was found compliant with Occupational Safety and Health Administration standards.

Isolation of CTPs From BMA

A standardized isolation protocol was used for all BMA (Figure 2). The BMA was filtered through a 70-micron mesh to remove blood clots. Before plating each sample, the total number of nucleated cells per 1.0 mL of BMA was counted and recorded using a Coulter counter (Beckman Coulter, Inc, Fullerton, California). Cell viability was assessed for each isolation procedure by trypan-blue exclusion using a hemocytometer.

In an attempt to maximize patient outcome and minimize anesthetic and surgery times, we sought to develop a rapid method of isolation of CTPs. Two commonly used methods were compared with this new method. Each BMA sample was divided into 3 groups:

Isolation 1 (OR): A new method, designed for use in the OR, consisting of one 5-minute centrifugation at 1500 rpm in which BMA was overlaid onto a 17.5% sucrose gradient in a 50-mL conical tube followed by extraction of CTPs in the fractionated layer.

Isolation 2 (Laboratory): A commonly used laboratory method to isolate CTPs in which a 30-minute centrifugation at 1500 rpm is followed by a fractionated layer extraction of CTPs using a Histopaque gradient (Sigma-Aldrich, St Louis, Missouri).[22,41]

Isolation 3 (Laboratory): A standard laboratory method for the isolation of CTPs that involves plating whole BM for 24 hours, aspirating nonadherent cells, and expanding the attached cells for surgical reimplantation.[10,17,26]

Cell Culture Conditions

Nucleated cells harvested from the fractionated layer in isolations 1 and 2 were counted and plated on 100-mm Primaria dishes (BD Laboratories, Franklin Lakes, New Jersey) at a concentration of 0.5×10^6cells/9.6 cm^2 into control media containing phenol red free α-minimum essential medium (Invitrogen, Carlsbad, California), 10% fetal bovine serum (Atlanta Biologicals, Atlanta, Georgia), and 0.1% penicillin/streptomycin (Invitrogen). For isolation 3 (laboratory), cells were counted to normalized for plating density and plated under the same conditions as in isolations 1 and 2. To remove nonadherent cells in isolation 3 (laboratory), media was aspirated after 24 hours of incubation.

To ensure that sterility was maintained, all cultures were checked daily for contamination. For all assays, six 100-mm dishes were plated per patient and for all 3 isolation methods for a total of 18. This was repeated for each patient, making a total of 414 dishes.

Flow Cytometric Analysis

Fluorescence-activated cell sorting (FACS) was used to ensure that cells obtained from the proximal humerus had surface markers characteristic of stem cells. Cells were grown in control media, trypsinized in 0.25% trypsin/EDTA at confluence, rinsed, and centrifuged. The pellet was resuspended in staining buffer containing 1% human serum, 1% bovine serum albumin, and 1% fetal bovine serum in phosphate-buffered saline. Cells were incubated with either phycoerythrin or fluorescein isothiocyanate antibodies, washed with staining buffer, and analyzed using a FACSCalibur (BD Biosciences, San Jose, California).

Phycoerythrin-conjugated mouse monoclonal anti-CD73 immunoglobulin G (IgG), anti-CD90 IgG, and fluorescein isothiocyanate-conjugated antimouse CD45 monoclonal IgG were obtained from BD Biosciences. All antibodies were reactive against human antigens. Testing with negative and positive controls confirmed the specificity of these antibodies.

Differentiation of CTPs Into Bone

To show that CTPs had the ability to differentiate into bone, cells were grown in osteogenic stem cell media (OSCM) containing control media, 50 μg/mL ascorbic acid, 4 mM β-glycerol phosphate, and 10^{-8} M dexamethasone (Sigma).[6] The number of CTPs in a sample that are capable of osteogenesis can be quantified by counting the number of colony-forming units (CFUs) expressing alkaline phosphatase (ALP) in culture (CTP-AP). Three parameters were measured directly or calculated from the results of cell culture: (1) the prevalence of CTPs (the number of CTP-AP/10^6 nucleated cells), (2) the concentration of CTPs (the number of CTP-AP/1.0 mL of BMA), and (3) the nucleated-cell count (the number of nucleated cells per 1.0 mL of BMA).

Quantification of Osteogenic CTP

Eight or more cells in a cluster were counted and defined as CFUs.[25] Those staining positive for ALP (90% or more in a CFU) were defined as CTP-AP. The ALP-positive cells in all groups were counted on day 7 to assess the number of osteogenic CTPs. Cells were fixed for 20 minutes in a 37% formaldehyde solution containing sodium citrate and acetone and incubated for 30 minutes in 0.2M Tris Buffer, pH 8.3, with AS-MX phosphate as a substrate and Fast Blue as a stain (Sigma). The ALP-positive cells stained blue/purple. The CTP cell concentration and prevalence were calculated with respect to the volume of BMA and the total number of nucleated cells.

Alkaline Phosphatase Staining

Evaluation of the osteogenic nature of CTPs giving rise to CFUs was performed with ALP staining of treated and untreated cells from isolation procedures 1 through 3 and was assessed at days 7, 14, and 21. Colonies expressing 95% to 100% ALP-positive cells were considered positive. Cells were fixed with an acetone/citrate/formalin solution using an ALP staining kit (Sigma), washed, and stained using Napthol AS-BI phosphate as substrate, and counterstained with neutral red.

RNA Isolation and Quantitative Polymerase Chain Reaction

Gene expression assays were performed by culturing cells for 7 and 14 days in OSCM. The RNA was isolated using Trizol reagent (Invitrogen). One microgram of RNA was reverse-transcribed using Reverse Transcriptase II kit and Oligo(dT) (Invitrogen). The cDNA was used in polymerase chain reactions (PCRs) containing a specific human primer (see online Appendix 1 for this article at http://ajs.sagepub.com/supplemental/) and SYBR-Green PCR master mix (Applied Biosystems, Foster City, California). The PCR reactions were performed in triplicate; the threshold cycles were obtained using Applied Biosystems software. Glyceraldehyde 3-phosphate dehydrogenase served as the endogenous control.

Statistical Analysis

Patient outcomes data were analyzed using 2-sample equal variance *t* test and Wilcoxon rank sum test to explore differences between the aspirate and control groups. Cellular data were analyzed using the Student *t* test and analysis of variance. The α-level for all statistics was set at .05.

RESULTS

Clinical Outcomes and Morbidity

There were 11 women and 12 men in both the aspirate and control groups. The mean ages in the aspirate and control groups were 56 ± 8.7 and 56 ± 8.9 years, respectively (see online Appendix 2 for this article at http://ajs.sagepub.com/supplemental/). The mean time to follow-up was 10.6 ± 6.7 months in the aspirate group and 10.0 ± 6.2 months in the control group. There were no surgical complications in any group. There was no incidence of RSD, DVT, wound irregularities, hematoma, or septic arthritis (0%). There was 1 superficial wound infection in the control group (4%) compared with zero in the aspirate group (0%). There was no incidence of abnormal pain in the aspirate group (0%) compared with 3 in the control group (13%). Two of these 3 patients had structural failures on postoperative MRI. There were 3 incidences of delayed or failed healing in the aspirate group (13%)

compared with 3 incidences in the control group (13%). Of the 3 incidences in the aspirate group, the first exhibited an avulsion fracture of the greater tuberosity on radiograph at postoperative day 9. Of note, there was a preexisting tuberosity fracture seen on preoperative radiographs that appeared to heal. The second incidence exhibited a partial-thickness rotator cuff tear on the operative side on MRI at 9 months after surgery. The third incidence was considered a functional failure because MRI at 6 months after surgery revealed an intact rotator cuff. In the control group, all 3 incidences of delayed or failed healing exhibited structural failure of the RCR on postoperative MRI. There were 2 incidences of stiffness in the aspirate group (8%) compared with 1 incident in the control group (4%). All incidences of stiffness were attributed to postoperative adhesive capsulitis.

There were no statistically significant differences in the SANE score (aspirate, 86.3 ± 10.5; control, 83.6 ± 15.1; $P = .54$) or postoperative forward elevation (aspirate, 163.0° ± 30.6°; control, 145.7° ± 41.4°; $P = .12$) and external rotation between the 2 groups ($P > .05$). The mean postoperative external rotation at the time of follow-up was 65.0° ± 20.4° in the aspirate group and 62.5° ± 17.1° in the control group ($P = .67$). There was no significant difference in postoperative strength between groups (median, 5; range, 4-5 in the aspirate group compared with median, 5; range, 4-5 in the control group; $P > .05$).

The remaining 43 patients (44 RCRs) were also evaluated to ensure adequate examination of morbidity. There were 16 women and 27 men with a mean age of 52.0 ± 9.5 years. The mean time to follow-up was 4.6 ± 2.9 months. There was no incidence of RSD, DVT, wound irregularities, or septic arthritis (0%). There were 5 instances of abnormal pain (12%), 7 instances of delayed or failed healing (16%), 3 instances of stiffness (7%), all of which were exhibited by postoperative adhesive capsulitis, and 1 instance of hematoma (2%) and 5 instances of abnormal pain (9%), of which 4 patients had failed healing and 1 had adhesive capsulitis. The mean SANE score at the time of follow-up was 77.5 ± 11.0. The mean forward elevation and external rotation at the time of follow-up were 147.9° ± 48.1° and 57.1° ± 33.8°, respectively. The median strength grade was 4.5 of 5.

Collection of BMA

Bone marrow was successfully aspirated in all 23 cases. The average volume of BM after 60 seconds of aspiration was 23.5 ± 7.5 mL (see online Appendix 2 for this article at http://ajs.sagepub.com/supplemental/). Although it has been shown that a volume greater than 2 mL of BM contains peripheral blood, it has also been shown that no one volume is optimum for the achievement of all of the possible goals of aspiration.[37] The intent of this study was to obtain a volume of BM for easier manipulation in the OR. The goal was to make the procedure easy for the staff to implement during arthroscopic surgery without any compromise in speed, sterility, or OR standards. No contamination was observed in any of the aspirates.

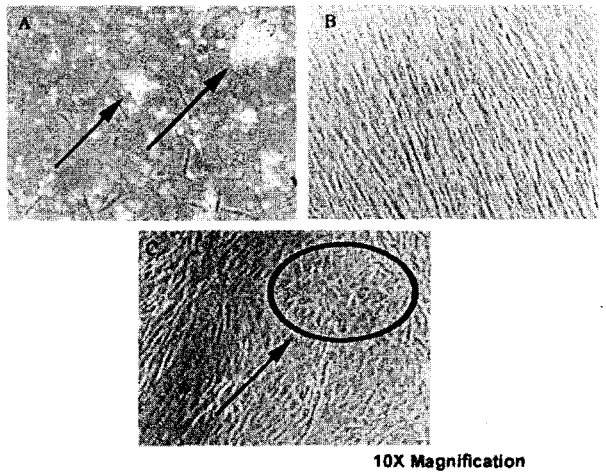

Figure 3. A, colony-forming units (indicated by arrows) after 48 hours cultured with control media. B, connective tissue progenitor cells grown for 7 days in control media align in a well-organized, parallel fashion. This observation is consistent with parental bone marrow stromal cells as described by Wang et al.[49] C, connective tissue progenitor cells cultured for 21 to 28 days in osteogenic media resulted in morphologic transformation from long spindle-shaped cells to a cuboidal shape, indicative of an osteoblastic morphology (magnification ×10).

Cell Morphology

After 7 days the attached cells from all isolation procedures produced CFUs, which proliferated quickly (Figure 3A). By days 21 to 28 the colonies grown in control media had formed a uniform multicellular layer that had spindle-shaped cells, which are comparable with progenitor cells found throughout the literature (Figure 3B).[29,30,39] Cells grown in OSCM resulted in a morphologic transformation from long spindle-shaped cells into a cuboidal-shaped cells, indicative of bone cells (Figure 3C).

FACS Analysis

The FACS analysis showed that CTPs grown in control media were positive for CD73 and CD90 and simultaneously negative for CD45 (Figure 4B-D), which is characteristic of adult mesenchymal stem cells.

Prevalence and Concentration of CTPs

The number of ALP-positive CFUs in BMA is determined by 2 variables: the number of nucleated cells and the prevalence of ALP-positive CFUs among the nucleated cells.[3,37] Before plating the cells, each BMA was counted to obtain nucleated cell numbers and was normalized to a specific plating density (0.5×10^6cells per well). Cell viability as assessed by trypan blue exclusion for isolation procedures 1, 2, and 3 was 95% ± 1.2%, 94% ± 1.4%, and 93% ± 2.5%, respectively. To

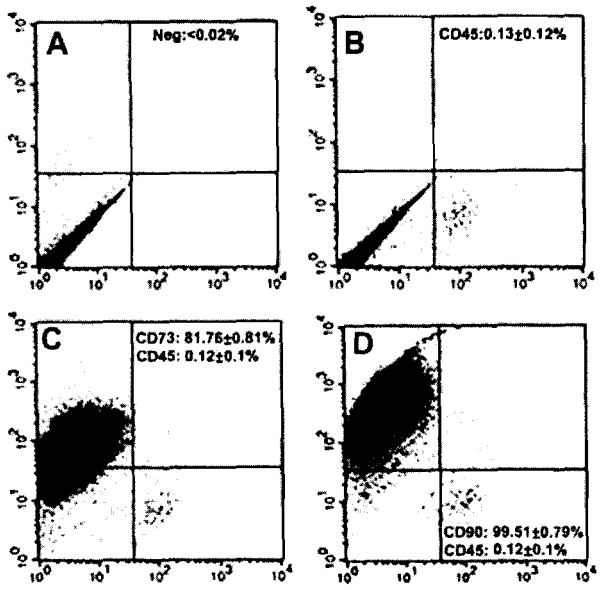

Figure 4. Flow cytometric analysis (FACS) of connective tissue progenitor cells. A, FACS analysis for a double-negative control: (-) phycoerythrin and (-) fluorescein isothiocyanate. B, the connective tissue progenitor cells (CTPs) were stained with fluorescein isothiocyanate-conjugated antimouse CD45 monoclonal immunoglobulin G, as a negative control. C and D, CTPs stained with CD73 and CD45 antibodies (C) and CD90 and CD45 antibodies (D). The average percentage of positive cells for each marker in 6 CTP populations is listed in each panel.

determine the prevalence and concentration of CTPs capable of osteogenesis in the BMA, cells from each patient were plated into OSCM.[6] On day 7 cells were stained for ALP expression, and colonies with 8 or more cells staining positive for ALP were counted (CTP-AP). The prevalence of CTPs was significantly increased for isolation 1 (OR) compared with isolations 2 and 3 (18.2 ± 0.95, 11.1 ± 1.4, and 7.2 ± 1.1 CTP-AP/million nucleated cells, respectively ($P < .05$) (Figure 5A). Furthermore, although variability with respect to prevalence of CTPs was noted for all patients, isolation 1 consistently showed a higher prevalence of CTPs compared with isolations 2 and 3 (Figure 6).

Differentiation of Isolated CTPs

Bone sialoprotein (BSP), ALP, and type I collagen are established bone markers.[6,44,48] Cells cultured in osteogenic differentiation media exhibited up-regulation of BSP, ALP, and type I collagen transcription levels between 7 and 14 days in culture. Cells harvested from the 5- and 30-minute isolations expressed significantly higher transcription levels of ALP and type I collagen as compared with the 24-hour plated whole marrow after 7 and 14 days in culture ($P < .05$) (Figure 7A). At day 14, type I collagen transcription was significantly higher in the 5-minute isolation than in the 30-minute and whole

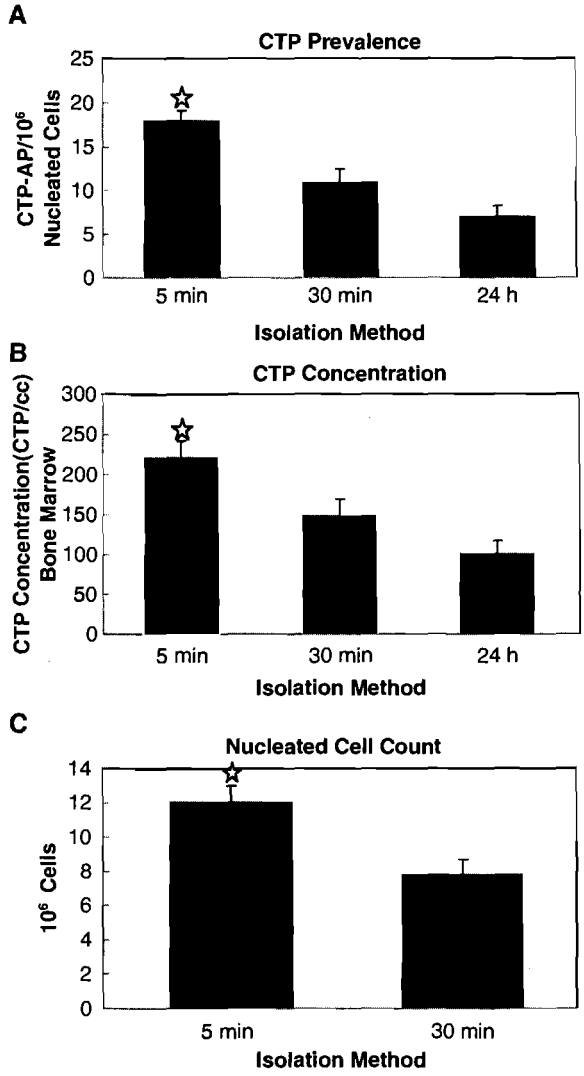

Figure 5. Comparison of 3 isolation techniques. A 5-minute centrifugation showed a significant increase in connective tissue progenitor cell prevalence (A) and concentration (B) compared with a 30-minute centrifugation or bone marrow aspirate plated for 24 hours. Nucleated cell number was significantly increased after a 5-minute centrifugation compared with a 30-minute spin (C). Stars represent significance between isolation methods ($P < .05$).

marrow isolations (Figure 7B). The BSP transcription levels were significantly increased at day 14 in the 5-minute isolation compared with isolations 2 and 3 (Figure 7C).

Alkaline Phosphatase Staining

Gene expression profiles were complemented by ALP staining of the cells after 7, 14, and 21 days in culture. Alkaline phosphatase staining is similar to ALP gene expression, with increased positive colonies at days 14 and 21 (Figure 8). Interestingly, the 30-minute isolation method showed

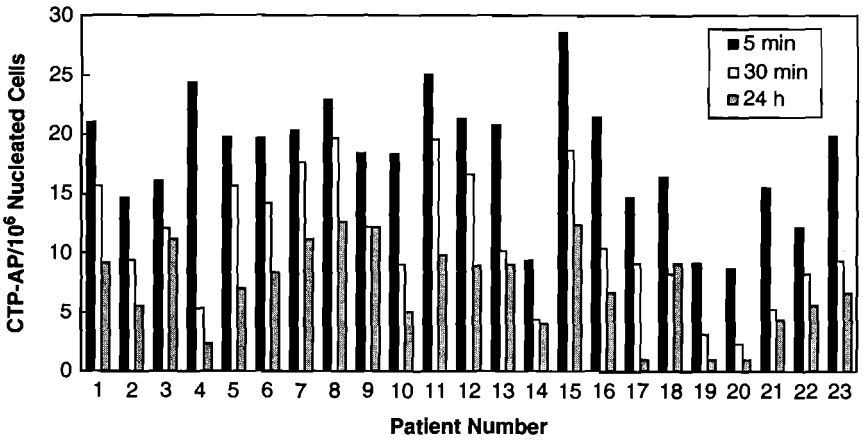

Figure 6. Prevalence of connective tissue progenitor cells in individual patients. The CTP concentration/1.0 mL of BMA was also significantly increased in isolation 1 (OR) (222.6 ± 19.3) compared with the 30-minute fractionated cells (150 ± 20.0) and the 24-hour plated whole marrow samples (102.0 ± 15.0, $P < .05$) (Figure 5B). For isolations 1 and 2, the number of nucleated cells before plating was 12.1 ± 0.86 × 10^6cells/mL and 7.9 ± 0.80 × 10^6 cells/mL of BMA (Figure 5C).

less ALP exhibiting cells than the 5-minute and 24-hour isolations.

DISCUSSION

In cases of massive RCR, bone-to-tendon healing is unpredictable.[14,16] Biologic augmentation may improve this healing process.[24,35,45,46] Early evidence indicates that adult stem cells are capable of regenerating diseased tissue.[2,8,12,40] The first purpose of this study was to determine if BM could be aspirated from the proximal humerus efficiently and without an increase in patient morbidity. We found no significant increases in the incidence of postoperative complications. To ensure we had adequately examined morbidity, we evaluated all remaining patients who underwent arthroscopic RCR in the study period. These were patients who did not undergo aspiration and were not matched to the control cohort. The results of this comparison did not demonstrate any significant increase in complications, further supporting the hypothesis that aspiration from the proximal humerus does not increase morbidity.

For consistency and reproducible surgical technique, the surgeon pulled back on the syringe to maximize suction for 60 seconds. This method yielded a mean of 23.5 mL of BM. Although it has been well documented that a volume greater than 2 mL of BM contains peripheral blood, a sample this small is difficult to manipulate in the real-time setting of the OR.[37] The intent of this study was to obtain a large volume of BM for easier manipulation in the OR. The goal was to make the procedure easy for the staff to implement during arthroscopic surgery without any compromise in speed, sterility, or standards. A larger volume of aspirate coincides with a larger fractionated layer and is ideal for potential future use with biologic scaffolds that often require saturation before being surgically introduced.

The second purpose of this study was to develop a simple and efficient method of extracting and purifying BM during arthroscopic rotator cuff surgery and to purify a fraction rich with CTPs. It was important that the extraction of BM did not conflict with the primary goal of repairing the rotator cuff. A 14-gauge needle was used in lieu of the traditional 8- to 11-gauge Jamshidi needle (Baxter Health Care Corp, Valencia, California). The size difference between the 14-gauge (inner diameter, 0.254 mm) compared with a 10-gauge (inner diameter, 0.356 mm) is significant. However, in the procedure presented, the needle gauge was used to act as a punch for the suture anchor for the RCR. Using a 14-gauge needle instead of an 8- to 11-gauge needle reduces patient morbidity in the arthroscopic rotator cuff procedure. Cell viability results show that this did not limit the harvest of CTPs.

Our goal was to develop a protocol for purifying BM that was safe, simple, reproducible, and efficient (isolation 1, OR). To evaluate this purification method, we chose 2 standard methods (laboratory isolation 2 and 3) for comparison. Isolation 2 (laboratory) required 30 minutes of centrifugation whereas isolation 3 (laboratory) required plating of BM for a 24-hour period. While both of these methods have been shown to reproducibly isolate CTPs, the time involved in each is prohibitive to the surgeon. Nucleated cell counts from isolation 1 (OR) compared with isolation 2 (laboratory) were greater, indicating that 5 minutes of centrifugation was adequate to produce a fraction rich in cells capable of forming colonies. The number of nucleated cells was determined to be 12.1 ± 0.86 cells/mL, which is comparable with nucleated cell counts observed in the literature for the vertebral body and iliac crest.[20,33] Further, untreated cells from all 3 isolation procedures produced spindle-shaped CFUs indicative of CTPs and were positive for cell surface markers characteristic of adult mesenchymal stem cells as shown by FACS analysis.[38] By day 28, treated cells had formed a uniform multicellular layer that had a cobblestone morphologic appearance indicative of bone cells.

This study used a sucrose gradient based on its current availability in the OR and its ease of sterility. The 17.5% sucrose gradient was made to mimic the density of the

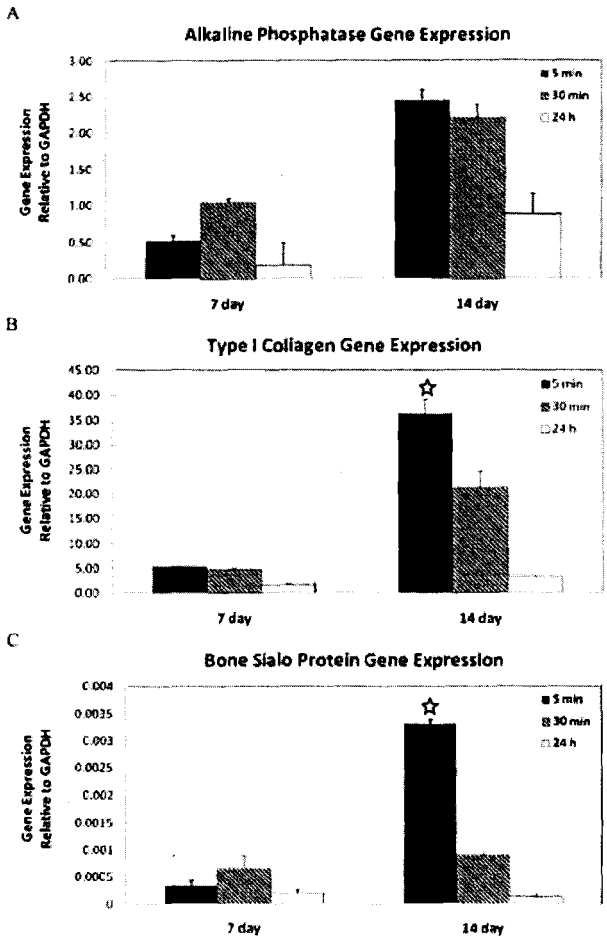

Figure 7. Osteogenic potential of connective tissue progenitor cells isolated by various methods. Connective tissue progenitor cells cultured in osteogenic media exhibited significantly higher transcription levels of the osteogenic markers alkaline phosphatase (ALP), bone sialoprotein, and type I collagen after 14 days in culture. Five-minute fractionation provided a significantly higher expression of ALP mRNA levels than the 30-minute fractionated samples and the 24-hour plated marrow after 2 weeks in culture (A). After 2 weeks in culture, type I collagen and bone sialoprotein transcription levels were significantly higher in the 5-minute isolation method compared with the 30-minute and 24-hour isolation methods (B and C). Stars represent significance between isolation methods ($P < .05$).

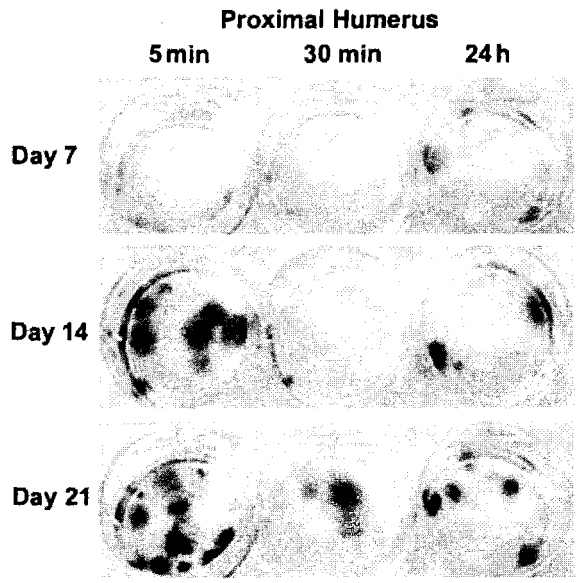

Figure 8. Alkaline phosphatase (ALP) staining for the osteogenic marker ALP at 7, 14, and 21 days shows increased positive colonies after 14 and 21 days in culture. Interestingly, however, cells harvested from the 30-minute fractionation exhibited less ALP staining than the cells adhering for 24 hours after 14 days in culture.

commercially available Ficoll-Paque gradient (1.077 g/mL), which is commonly used for the isolation of CTPs.[21,22] The osmolarity of the Ficoll-Paque gradient is 299 mOsm/L and the osmolarity of the sucrose gradient used in our study was determined to be 501 mOsm/L.[21] Because high osmolarity is capable of inducing cellular lysis, cell death was initially a concern. The comparison of the 5- and 30-minute centrifugations demonstrated no statistically significant differences, indicating that the use of a sucrose gradient for 5 minutes is an acceptable method to obtain high numbers of viable cells in a clinical setting. Trypan blue exclusion demonstrated a mean cell viability of 95% after the use of a sucrose gradient, which was not statistically different from the other isolation procedures, further supporting its clinical applicability.

The third purpose of this study was to determine if the fractionated layer contained CTPs that could be purified and differentiated into bone. Bone was chosen for its involvement in the tendon-to-bone reinsertion during massive RCRs and the large amount of literature supporting the differentiation of CTPs into osteoblasts. The osteogenic potential of marrow-derived CTPs is determined by the occurrence of ALP-positive CFU among nucleated cells. Alkaline phosphatase is a glycoprotein that has a high activity in mineralizing bone, where it is mainly localized in the plasma membrane of osteoblastic cells.[11,28] After 7 days of treatment in osteogenic media, cells demonstrated the formation of ALP-positive CFUs from each of the isolation procedures. The number of CFUs expressing ALP per one million nucleated cells after a 5-minute isolation was significantly higher than cells isolated from a 30-minute centrifugation, suggesting that the proximal humerus is a suitable site for the isolation of CTPs.

Gene expression was examined to further determine the osteogenic potential of these CTPs. Before gene expression analysis, equal amounts of RNA were used to normalize for cell density. Glycoproteins BSP, ALP, and type I collagen gene expression levels are up-regulated in an osteoblastic phenotype.[1] At low concentrations, dexamethasone has been shown to induce mRNA levels of osteoblastic markers in BM-derived CTPs.[4,17,43,51] Both 5- and 30-minute isolation methods produced higher transcription levels of ALP,

type I collagen, and BSP between 7 and 14 days. In addition, these 2 isolation techniques produced significantly higher transcription levels of ALP, type I collagen, and BSP at 14 days compared with the 24-hour isolation. This gene expression profile was complemented with positive ALP staining of these cells after 7 and 14 days in culture. These results show that CTPs isolated using a 5-minute centrifugation exhibit osteogenic potential similar to other accepted isolation procedures (isolations 2 and 3).

A limitation of this study is that CTPs were only differentiated into an osteogenic lineage. Differentiation into other mesenchymal cells would have strengthened this study. Our intention was to develop a method of extracting and purifying BM to give the orthopaedic surgeon a simple, safe, and efficient way to isolate CTP during arthroscopic RCR surgery. The proposed method of aspiration did not result in an increase in complications. The BMA was successfully purified in the OR, with no resulting increase in operation time, and a fractionated layer rich with CTP was successfully obtained. Furthermore, these cells demonstrated osteogenic potential evidenced by differentiation into osteoblasts. The results of this study may serve as an initial step toward enhancing the healing process in the surgical repair of massive rotator cuff tears.

REFERENCES

1. Aubin JE. Advances in the osteoblast lineage. *Biochem Cell Biol.* 1998;76(6):899-910.
2. Ballas CB, Zielske SP, Gerson SL. Adult bone marrow stem cells for cell and gene therapies: implications for greater use. *J Cell Biochem Suppl.* 2002;38:20-28.
3. Bauer TW, Muschler GF. Bone graft materials: an overview of the basic science. *Clin Orthop Relat Res.* 2000;371:10-27.
4. Beresford JN, Joyner CJ, Devlin C, Triffitt JT. The effects of dexamethasone and 1,25-dihydroxyvitamin D3 on osteogenic differentiation of human marrow stromal cells in vitro. *Arch Oral Biol.* 1994;39(11):941-947.
5. Caplan AI. The mesengenic process. *Clin Plast Surg.* 1994;21(3):429-435.
6. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. *Endocrinology.* 1994;134(1):277-286.
7. Cole BJ, ElAttrache NS, Anbari A. Arthroscopic rotator cuff repairs: an anatomic and biomechanical rationale for different suture-anchor repair configurations. *Arthroscopy.* 2007;23(6):662-669.
8. Corsi KA, Schwarz EM, Mooney DJ, Huard J. Regenerative medicine in orthopaedic surgery. *J Orthop Res.* 2007;25(10):1261-1268.
9. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol.* 2000;28(8):875-884.
10. Dennis JE, Haynesworth SE, Young RG, Caplan AI. Osteogenesis in marrow-derived mesenchymal cell porous ceramic composites transplanted subcutaneously: effect of fibronectin and laminin on cell retention and rate of osteogenic expression. *Cell Transplant.* 1992;1(1):23-32.
11. Doty SB, Schofield BH. Enzyme histochemistry of bone and cartilage cells. *Prog Histochem Cytochem.* 1976;8(1):1-38.
12. Eberli D, Atala A. Tissue engineering using adult stem cells. *Methods Enzymol.* 2006;420:287-302.
13. Fealy S, Rodeo SA, MacGillivray JD, Nixon AJ, Adler RS, Warren RF. Biomechanical evaluation of the relation between number of suture anchors and strength of the bone-tendon interface in a goat rotator cuff model. *Arthroscopy.* 2006;22(6):595-602.
14. Gerber C, Fuchs B, Hodler J. The results of repair of massive tears of the rotator cuff. *J Bone Joint Surg Am.* 2000;82(4):505-515.
15. Goutallier D, Postel JM, Bernageau J, Lavau L, Voisin MC. Fatty muscle degeneration in cuff ruptures: pre- and postoperative evaluation by CT scan. *Clin Orthop Relat Res.* 1994;304:78-83.
16. Grana WA, Teague B, King M, Reeves RB. An analysis of rotator cuff repair. *Am J Sports Med.* 1994;22(5):585-588.
17. Grigoriadis AE, Heersche JN, Aubin JE. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *J Cell Biol.* 1988;106(6):2139-2151.
18. Harryman DT 2nd, Mack LA, Wang KY, Jackins SE, Richardson ML, Matsen FA 3rd. Repairs of the rotator cuff: correlation of functional results with integrity of the cuff. *J Bone Joint Surg Am.* 1991;73(7):982-989.
19. Hayes K, Walton JR, Szomor ZR, Murrell GA. Reliability of five methods for assessing shoulder range of motion. *Aust J Physiother.* 2001;47(4):289-294.
20. Hernigou P, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions: influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am.* 2005;87(7):1430-1437.
21. Hosseini JM, Yang XY, Elin RJ. Determination of magnesium in granulocytes. *Clin Chem.* 1989;35(7):1404-1407.
22. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem.* 1997;64(2):295-312.
23. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res.* 1998;238(1):265-272.
24. Kovacevic D, Rodeo SA. Biological augmentation of rotator cuff tendon repair. *Clin Orthop Relat Res.* 2008;466(3):622-633.
25. Krebsbach PH, Kuznetsov SA, Bianco P, Robey PG. Bone marrow stromal cells: characterization and clinical application. *Crit Rev Oral Biol Med.* 1999;10(2):165-181.
26. Lennon DP, Edmison JM, Caplan AI. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. *J Cell Physiol.* 2001;187(3):345-355.
27. Liu SH, Baker CL. Arthroscopically assisted rotator cuff repair: correlation of functional results with integrity of the cuff. *Arthroscopy.* 1994;10(1):54-60.
28. Majeska RJ, Rodan GA. Alkaline phosphatase inhibition by parathyroid hormone and isoproterenol in a clonal rat osteosarcoma cell line: possible mediation by cyclic AMP. *Calcif Tissue Int.* 1982;34(1):59-66.
29. Majumdar MK, Banks V, Peluso DP, Morris EA. Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. *J Cell Physiol.* 2000;185(1):98-106.
30. Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J Cell Physiol.* 1998;176(1):57-66.
31. Mazzocca AD, Millett PJ, Guanche CA, Santangelo SA, Arciero RA. Arthroscopic single-row versus double-row suture anchor rotator cuff repair. *Am J Sports Med.* 2005;33(12):1861-1868.
32. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell.* 2004;6(4):483-495.
33. McLain RF, Fleming JE, Boehm CA, Muschler GF. Aspiration of osteoprogenitor cells for augmenting spinal fusion: comparison of progenitor cell concentrations from the vertebral body and iliac crest. *J Bone Joint Surg Am.* 2005;87(12):2655-2661.
34. Mirowitz SA. Hematopoietic bone marrow within the proximal humeral epiphysis in normal adults: investigation with MR imaging. *Radiology.* 1993;188(3):689-693.
35. Moffat KL, Kwei AS, Spalazzi JP, Doty SB, Levine WN, Lu HH. Novel nanofiber-based scaffold for rotator cuff repair and augmentation. *Tissue Eng Part A.* 2009;15(1):115-126.

36. Murray TF Jr, Lajtai G, Mileski RM, Snyder SJ. Arthroscopic repair of medium to large full-thickness rotator cuff tears: outcome at 2- to 6-year follow-up. *J Shoulder Elbow Surg*. 2002;11(1):19-24.
37. Muschler GF, Boehm C, Easley K. Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. *J Bone Joint Surg Am*. 1997;79(11):1699-1709.
38. Nesti LJ, Jackson WM, Shanti RM, et al. Differentiation potential of multipotent progenitor cells derived from war-traumatized muscle tissue. *J Bone Joint Surg Am*. 2008;90(11):2390-2398.
39. Owen M. Marrow stromal stem cells. *J Cell Sci Suppl*. 1988;10: 63-76.
40. Pittenger M, Vanguri P, Simonetti D, Young R. Adult mesenchymal stem cells: potential for muscle and tendon regeneration and use in gene therapy. *J Musculoskelet Neuronal Interact*. 2002;2(4): 309-320.
41. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284(5411): 143-147.
42. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*. 1997;276(5309):71-74.
43. Rickard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC, Riggs BL. Isolation and characterization of osteoblast precursor cells from human bone marrow. *J Bone Miner Res*. 1996;11(3):312-324.
44. Risteli L, Risteli J. Biochemical markers of bone metabolism. *Ann Med*. 1993;25(4):385-393.
45. Rodeo SA. Biologic augmentation of rotator cuff tendon repair. *J Shoulder Elbow Surg*. 2007;16(5 Suppl):S191-197.
46. Rodeo SA, Potter HG, Kawamura S, Turner AS, Kim HJ, Atkinson BL. Biologic augmentation of rotator cuff tendon-healing with use of a mixture of osteoinductive growth factors. *J Bone Joint Surg Am*. 2007;89(11):2485-2497.
47. Sordella R, Jiang W, Chen GC, Curto M, Settleman J. Modulation of Rho GTPase signaling regulates a switch between adipogenesis and myogenesis. *Cell*. 2003;113(2):147-158.
48. Stein GS, Lian JB. Molecular mechanisms mediating proliferation/ differentiation interrelationships during progressive development of the osteoblast phenotype. *Endocr Rev*. 1993;14(4):424-442.
49. Wang QW, Chen ZL, Piao YJ. Mesenchymal stem cells differentiate into tenocytes by bone morphogenetic protein (BMP) 12 gene transfer. *J Biosci Bioeng*. 2005;100(4):418-422.
50. Williams GN, Gangel TJ, Arciero RA, Uhorchak JM, Taylor DC. Comparison of the Single Assessment Numeric Evaluation method and two shoulder rating scales: outcomes measures after shoulder surgery. *Am J Sports Med*. 1999;27(2):214-221.
51. Yamanouchi K, Satomura K, Gotoh Y, et al. Bone formation by transplanted human osteoblasts cultured within collagen sponge with dexamethasone in vitro. *J Bone Miner Res*. 2001;16(5):857-867.
52. Zheng N, Harris HW, Andrews JR. Failure analysis of rotator cuff repair: a comparison of three double-row techniques. *J Bone Joint Surg Am*. 2008;90(5):1034-1042.