Collagenous Microbeads as a Scaffold for Tissue Engineering with Adipose-Derived Stem Cells

J. Peter Rubin, M.D.
Jennifer M. Bennett, M.S.
John S. Doctor, Ph.D.
Bradley M. Tebbets, B.S.
Kacey G. Marra, Ph.D.

Pittsburgh, Pa.

Background: Standard approaches to soft-tissue reconstruction include autologous tissue flaps and alloplastic implants. Both of these approaches have disadvantages, including donor-site morbidity, implant migration, and foreign body reaction. Autologous fat transplantation, with a minimally invasive cannula harvest, has lower donor-site morbidity than tissue flaps do, but there is an unpredictable degree of resorption of the transplanted fat over time. Adipose-derived stem cells isolated from harvested fat are better able to withstand the mechanical trauma from the suction cannula and may allow for improved cell survival and generation of new fat tissue after transfer to another anatomic site. The authors hypothesized that porous collagenous microbeads (CultiSphers; Sigma, St. Louis, Mo.) could be useful as injectable cell delivery vehicles for adipose-derived stem cells. This strategy would allow induction of differentiation ex vivo and precise placement of cells and scaffold in a tissue bed. The objective of this study was to assess the ability of the stem cells to proliferate and differentiate on these microbeads.

Methods: Adipose-derived stem cells were isolated from discarded human adipose tissue and cultured on porous collagenous microbeads in a stirred bioreactor (spinner flask). The cells attached and proliferated on the microbeads and maintained high viability over several weeks of culture.

Results: When exposed to adipogenic or osteogenic medium, the cells differentiated into adipocytes and osteoblasts, respectively, while attached to the microbeads.

Conclusion: Collagenous microbeads are a favorable scaffold for adipose-derived stem cells, allowing ex vivo proliferation and differentiation on particles that are small enough to be injected. (*Plast. Reconstr. Surg.* 120: 414, 2007.)

Mesenchymal stem cells isolated from adipose tissue are plentiful, can be harvested with low morbidity, and have the potential to differentiate into multiple mature tissue types.[1] The mesenchymal stem cells derived from adipose tissue can be maintained for extended periods of time in culture, have a mesenchyme-like morphology, and demonstrate plasticity and multilineage potential in vitro,[1–8] including myogenic,[1] osteogenic,[8] neuronal,[3,4] leiomyogenic,[7] and cardiomyocytic differentiation.[5] Suitable scaffold materials for use with these cells should provide a favorable surface for adherence, proliferation, and differentiation while lending structural support to the developing mass of new tissue. CultiSphers (Sigma, St. Louis, Mo.) are commercially available, porous, collagenous microbeads with a diameter ranging between 100 and 380 μm and an average pore size of 20 μm. These microbeads may represent a clinically useful scaffold for adipose-derived stem cells, because they can be injected into a defect and molded into the desired shape without migration of the cells. The cell-seeded microbeads can be injected through a needle into the wound site, and agglomeration of the microbeads can retain the cells and microbeads in the site. Unlike hydrogels, collagenous microbeads allow immediate exposure of the adipose-derived stem cells to nutrients in the interstitial fluid after injection.

From the Division of Plastic Surgery, Department of Surgery, and Department of Bioengineering, University of Pittsburgh; Department of Biological Sciences, Duquesne University; and McGowan Institute for Regenerative Medicine.
Received for publication August 2, 2006; accepted January 3, 2007.

DOI: 10.1097/01.prs.0000267699.99369.a8

The type I collagen used to produce the microbeads has a history of successful use as an implant material in humans, and the beads can be modified to include growth factors.

The purpose of this study was to assess the ability of human adipose-derived stem cells to adhere to collagenous CultiSphers and proliferate in culture. In addition, we determined the capacity of the stem cells to differentiate into two cell types, adipocytes and osteoblasts, while adhering to CultiSphers and being cultured under defined conditions.

MATERIALS AND METHODS

This study was conducted in accordance with the regulations of the Human Studies Committee of the University of Pittsburgh. Tissue collection was performed according to a protocol approved by the University of Pittsburgh's Institutional Review Board.

Isolation of Human Adipose-Derived Stem Cells

The stem cells were extracted from human subcutaneous adipose tissue harvested during elective abdominoplasty. The adipose tissue was immediately transferred to the laboratory in phosphate-buffered saline supplemented with 1% penicillin/streptomycin and 1% amphotericin B. The fat tissue was then minced and digested in phosphate-buffered saline containing 1 mg/ml collagenase and 3.5% fatty acid–free bovine serum albumin in a 37°C shaking water bath until the mixture was homogeneous. The digests were then filtered through a double-layered gauze filter (350 μM) and centrifuged at 1000 rpm for 10 minutes. After centrifugation of the dispersed tissue (1000 rpm for 10 minutes at 21°C) and removal of the supernatant, the pellet was resuspended in 20 ml of erythrocyte lysis buffer (10 mM $KHCO_3$, 1 mM EDTA, and 154 mM NH_4Cl) for 10 minutes at room temperature, followed by another centrifugation step (1000 rpm for 10 minutes at 21°C). The cells were then transferred to plating medium (1:1 Dulbecco's modified Eagle medium/F12 containing 10% fetal bovine serum and antibiotics) at a density of 5×10^4 cells/ml. The adipose-derived stem cells adhered to tissue-treated T-flasks for 6 hours. Nonadherent cells and floating cells were removed by rinsing the cell culture with phosphate-buffered saline and covering it with the medium described above. All of the stem cells used in this study were from the same adipose depot from a single human subject (abdominal subcutaneous tissue, superficial to Scarpa's fascia).

Seeding of Adipose-Derived Stem Cells on Collagen Carriers

Porous collagenous beads with a diameter ranging from of 100 to 380 μm (CultiSphers-G or GL; Sigma) (Fig. 1) were sterilized by autoclaving the CultiSphers in phosphate-buffered saline (catalogue no. 14190-144; Gibco, Grand Island, N.Y.). To prepare each culture flask, CultiSphers (0.2 g) were washed, resuspended in 40 ml of plating medium (Dulbecco's modified Eagle medium/F12 with 10% fetal bovine serum) with 2×10^6 adipose-derived stem cells in a sterile spinner flask (catalogue no. 1967-00100; Bellco Glass, Vineland, N.J.), and incubated at 37°C with 5% CO_2 on a stir plate set to rotate at 15 rpm. The cells were allowed to attach for 24 hours. To remove unattached cells, CultiSphers were allowed to settle and were then washed four times with 15 ml of culture medium. CultiSphers, with attached cells, were then resuspended in 40 ml of culture medium and returned to a sterile spinner flask. Every 2 to 3 days, the spinner flask was removed from the stir plate, the CultiSphers were allowed to settle, and half of the medium was removed and replaced with fresh medium.

Assessment of Attachment and Proliferation of Adipose-Derived Stem Cells on CultiSphers

4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) stain was used to assess attachment and proliferation of the stem cells on the CultiSphers. One-milliliter samples were taken in triplicate from each spinner flask for DAPI staining. Each sample was washed three times in 2 ml of phos-

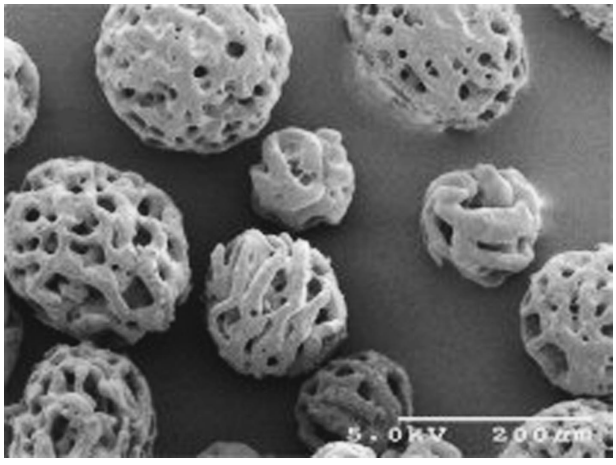

Fig. 1. Scanning electron microscopy image of collagenous beads (CultiSphers; Sigma, St. Louis, Mo.). Their porous structure provides a surface area for cell attachment and allows adequate exposure of cells to surrounding medium.

phate-buffered saline and fixed with 1.5 ml of 10% buffered formalin for 10 minutes. Samples were then washed four times with 2 ml of phosphate-buffered saline and incubated with 2 ml of DAPI (0.6 μg/ml in phosphate-buffered saline) for 10 minutes at room temperature, followed by four washes with 2 ml of phosphate-buffered saline. Fluorescence microscopy allowed visualization and counting of individual cell nuclei on CultiSphers. For determination of stem cell attachment during the 24-hour seeding period, samples were collected for DAPI staining at 0, 0.5, 1, 2, 4, 8, and 24 hours. For assessment of proliferation, samples were removed serially over a period of 49 days from spinner flasks, with cells maintained in plating medium.

Collagenase Treatment/Cell Viability

Cell viability of the adipose-derived stem cells in CultiSphers was quantified using collagenase digestion of CultiSphers and cell counting. Three 1-ml samples were removed from each spinner culture for collagenase treatment. Collagenase type II (0.17 g/100 ml of phosphate-buffered saline; catalogue no. LS004176, Worthington Biochemical, Lakewood, N.J.) was added to each sample and incubated in a 37°C water bath for 1 hour, or until the CultiSphers were digested. The digested samples were then centrifuged at 1000 rpm for 5 minutes to collect the released cells. The supernatant was removed and the pellet was resuspended in 100 μl of phosphate-buffered saline. A hemacytometer and trypan blue dye (Sigma T-8154) were used to count viable stem cells. The average number of viable cells per milliliter was then compared with the total number of CultiSphers present in 1 ml of suspension, to calculate the average number of viable stem cells per CultiSpher. The Live/Dead Viability/Cytotoxicity Assay Kit (Invitrogen Corp., Carlsbad, Calif.) was also used to evaluate the presence of live or dead stem cells on CultiSphers. One-milliliter samples were removed from spinner culture, the CultiSphers were washed three times with 1.5 ml of phosphate-buffered saline, and then cells on CultiSphers were stained, as directed by the supplier, with 2 μM calcein AM for live cells and 4 μM ethidium homodimer-1 for dead cells in 2 ml of phosphate-buffered saline.

Differentiation of Adipose-Derived Stem Cells to Adipocytes and Osteoblasts on CultiSphers

After 7 days of incubation, cells on CultiSphers in spinner flasks were subsequently cultured in adipocyte differentiation medium (Dulbecco's modified Eagle medium/F12, 33 μM biotin, 0.5 μM insulin, 17 μM pantothenate, 0.2 nM dexamethasone, 0.5 μM Ciglitazone (Biomol International, Plymouth Meeting, Pa.), 0.2 nM T3, 10 mg/ml transferrin, 540 μM 3-isobutyl-1-methylxanthine for 2 days, and antibiotics) for an additional 21 days. Spinner flasks in the control group were maintained with medium that did not include the adipogenic growth factors. As a positive control, stem cells cultured in standard wells without CultiSphers (at confluence) were treated with adipogenic medium and cultured for 21 days.

A separate experimental group was planned for osteoblast differentiation. After 7 days of incubation, cells on CultiSphers in spinner flasks were subsequently cultured in osteogenic supplement medium (PT-4120, which includes 100 nM dexamethasone, 10 mM beta-glycerophosphate, and 50 μg/ml ascorbic acid-2-phosphate; Cambrex, East Rutherford, N.J.) for an additional 26 days. Spinner flasks in the control group were maintained with medium that did not include the osteogenic growth factors.

Oil Red O Staining

To assess the adipocyte phenotype, Oil Red O (Sigma 00625) was used to stain for lipid vacuoles in differentiated cells.[9] Briefly, the CultiSphers were washed twice with phosphate-buffered saline and fixed with 10% buffered formalin (Fisher Scientific, Waltham, Mass.) for 10 minutes at room temperature. Cells were then washed twice with water before they were stained for 2 hours with the Oil Red O solution, and were then washed again.

Alkaline Phosphatase Histochemical Stain

To assess osteoblast differentiation, alkaline phosphatase histochemical stain (Sigma 85L-3R) was used to qualitatively detect the presence of alkaline phosphatase activity. Samples were removed from spinner flasks, washed three times with phosphate-buffered saline, fixed in 80% methanol/20% citrate working solution for 1 minute, and then washed four times with distilled water. Stain was then added, with incubation in the dark at room temperature for 2 hours. After incubation, the samples are washed three times with phosphate-buffered saline and viewed with bright field optics to observe alkaline phosphatase staining.

Alkaline Phosphatase Biochemical Assay

Triplicate samples of cells/CultiSphers were removed from spinner flasks, washed three times

with phosphate-buffered saline, and stored frozen in 250 μl of phosphate-buffered saline. To prepare cell lysates, samples were subjected to several freeze/thaw cycles. Production of p-nitrophenol was quantified using Lowry et al.'s method,[10] with comparison to a p-nitrophenol standard (Sigma 104-1) and absorbance quantitated at 405 nm. To normalize for the number of cells in each extract, protein determinations were performed on the lysates generated above using the BCA Protein Assay (Pierce Biotechnology, Rockford, Ill.).

Calcium Determination

Triplicate samples of cells/CultiSphers were removed from spinner flasks and washed three times with calcium-free phosphate-buffered saline. Calcium in each sample was solubilized in 0.5 ml of 0.1 N HCl. The calcium content of the acid extract was assessed colorimetrically using Sigma Calcium Kit 587-M and compared with a calcium standard.

Scanning Electron Microscopy

Samples of CultiSphers from spinner cultures were washed with phosphate-buffered saline and fixed in 2.5% glutaraldehyde (Sigma) in phosphate-buffered saline. The CultiSphers were stained with osmium tetroxide, dehydrated through an ethanol series, and dried to a critical point. Samples were coated with gold and viewed with a Hitachi 2460 scanning electron microscope at an accelerating voltage of 5 kV or 15 kV; digital images were captured at varying magnifications.

Statistical Analysis

The results are presented as mean ± SEM. Unpaired *t* tests were performed to assess differences in the adhesion and proliferation studies. Two-way analysis of variance was also used to further identify variations among treatment groups during differentiation. Statistical significance was set at a *p* value of less than or equal to 0.05.

RESULTS

Adipose-Derived Stem Cells Attach to CultiSphers and Proliferate in Culture

At the density of cells and CultiSphers described in the Materials and Methods section, the average number of cells adherent to each collagenous bead after 24 hours in the spinner flask was 2.4 ± 0.8 (Fig. 2). The stem cells proliferated in culture, with their number increasing to 5.9 ± 1.3 cells per bead after 7 days and 13.9 ± 1.8 cells per bead after 14 days. Proliferation continued over the 49-day observation period, reaching 51.4 ± 5.7 cells per bead at the last time point (Figs. 3 and 4).

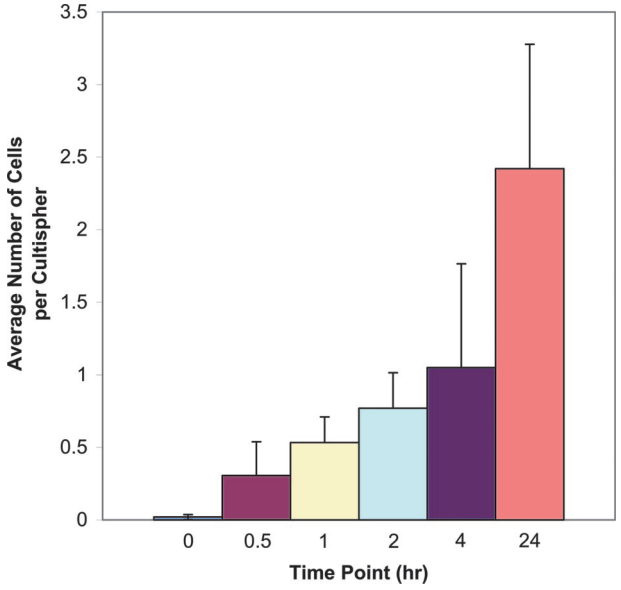

Fig. 2. Attachment of adipose-derived stem cells to CultiSphers. Samples were visualized by fluorescence microscopy. Cell nuclei on CultiSphers were then stained with DAPI and counted by inspection under the microscope. The number of cells per CultiSpher is reported (n = 100 CultiSphers, ±SD).

Adipose-Derived Stem Cells Differentiate to Adipocytes on CultiSphers

Adipose-derived stem cells attached to CultiSphers demonstrated accumulation of lipid by Oil Red O staining when exposed to adipogenic medium. The percentage of stem cells bound to CultiSphers with lipid accumulation was 12, 21, and 27 percent at 7, 14, and 21 days, respectively, after culture in adipogenic medium. Cells exposed to adipogenic medium in mass culture showed a higher percentage of differentiation (Table 1 and Fig. 5). No cells in the control group (plating medium) showed lipid accumulation.

Adipose-Derived Stem Cells Cultured on CultiSphers Express Alkaline Phosphatase and Deposit Calcium

To assess the ability of stem cells on CultiSphers to express markers for osteoblast differentiation, stem cells were seeded onto CultiSphers, as described in the Materials and Methods section, and cultured in spinner flasks for 1 week in mesenchymal stem cell growth medium. Culture was then continued with either osteogenic supplement–containing medium or mesenchymal stem cell growth medium as a control. Alkaline phos-

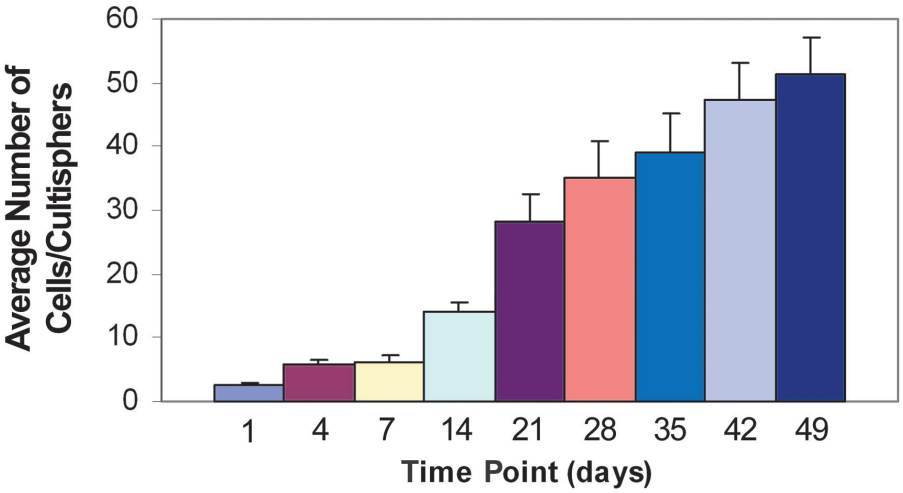

Fig. 3. Proliferation of adipose-derived stem cells to CultiSphers. Samples were visualized by fluorescence microscopy, and cell nuclei on CultiSphers were stained with DAPI and counted by inspection under the microscope. The number of cells per CultiSpher is reported ($n = 100$ CultiSphers, $\pm$SD).

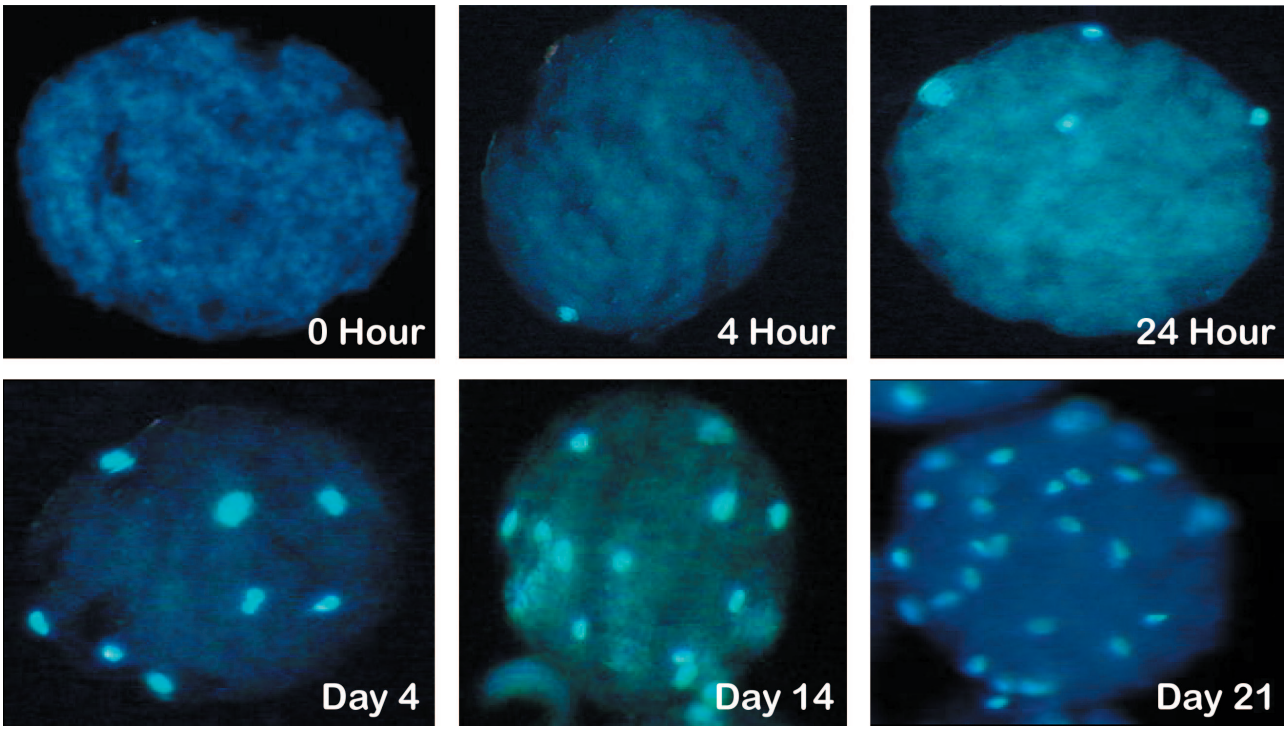

Fig. 4. Fluorescent images of the DAPI stain at time 0, 4 hours, 1 day, 4 days, 14 days, and 21 days after seeding with cells. CultiSphers are shown with small, bright nuclei evident.

phatase activity was assessed by both biochemical assay (Table 2) and histochemical staining (Fig. 6). Biochemical assays for alkaline phosphatase at 18 and 26 days indicated that there was no statistically significant difference ($p > 0.05$), based on analysis of variance and Tukey's honestly significant difference post hoc test, between control and osteogenic supplement–positive samples at either day 18 or day 26. There was a statistically significant increase in alkaline phosphatase ($p < 0.01$) between day 18 and day 26 for both treatments. In agreement with the biochemical assays, histochemical staining showed that alkaline phosphatase–positive cells were detected on CultiSphers cul-

Table 1. Differentiation of Adipose-Derived Stem Cells to Adipocytes on CultiSphers*

Treatment	Culture Type	Time Point (days)	Percent Differentiation
Plating medium	Spinner flask	7	0
		14	0
		21	0
Adipocyte differentiation medium	Spinner flask	7	13
		14	21
		21	27
Adipocyte differentiation medium	Stationary	7	21
		14	37
		21	59

*Differentiation of adipocyte-derived stem cells into adipocytes was assessed in four experimental groups: undifferentiated stem cells (plating medium only), differentiated stem cells in spinner flasks (adipocyte differentiation medium), osteogenic supplement–differentiated stem cells (osteogenic supplement medium), and differentiated stem cells in standard stationary culture (adipocyte differentiation medium). After 1 week of incubation in normal plating medium, the medium was changed to adipogenic differentiation medium. Oil Red O was used to stain for adipocytes. The stained adipocytes on the CultiSphers were captured using a digital camera, and the percentage of differentiated stem cells on the CultiSphers was determined by counting the positively stained cells and determining the ratio of stained cells to unstained cells (all cell nuclei were stained with DAPI).

tured in both control and osteogenic supplement medium (Fig. 6). Scoring of individual CultiSphers for alkaline phosphatase–positive cells using phase contrast microscopy revealed that 39 percent of the CultiSphers cultured in control medium had alkaline phosphatase–positive cells, compared with 33 percent of the CultiSphers in osteogenic supplement medium. In osteogenic supplement medium, approximately 50 percent of the CultiSphers were opaque, presumably due to deposition of a calcium-rich matrix, precluding the scoring of alkaline phosphatase–positive cells on these CultiSphers. No opaque CultiSphers were observed in control cultures.

Chemical determination of calcium at days 18 and 26 revealed a statistically significant increase in calcium deposition by adipose-derived stem cells on CultiSphers cultured in osteogenic supplement medium compared with control cultures (Table 3). Consistent with this osteogenic supplement–induced increase in calcium deposition, scanning electron microscopic observation revealed numerous cells on CultiSphers in both control and osteogenic supplement cultures, but structures characteristic of calcium phosphate deposition were present only in osteogenic supplement cultures (Fig. 7). Throughout the long period of culture in both control and osteogenic supplement medium, cells remained attached to and viable on CultiSphers (Fig. 8).

DISCUSSION

Therapeutic options for soft-tissue reconstruction include autologous tissue flaps (either pedicled or with microvascular anastomosis), prosthetic implants, dermal and fat grafting, and biomaterials derived from mammalian tissue, such

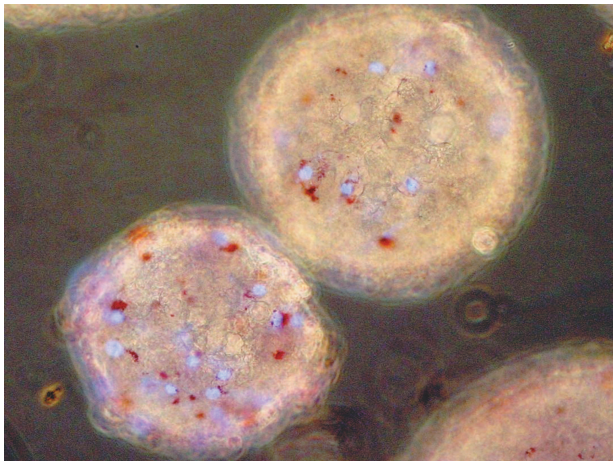

Fig. 5. Oil Red O–positive stain of adipose-derived stem cells in CultiSphers after 14 days in adipogenic medium, and nuclei counterstained with DAPI.

Table 2. Alkaline Phosphatase Activity of Adipose-Derived Stem Cells on CultiSphers*

	Day 18	Day 26
	(pmol p-nitrophenol phosphate/μg protein/60 min)	
No OS	98 ± 9†	484 ± 34
OS-positive	83 ± 10†	398 ± 16

OS, osteogenic supplement.
*Adipose-derived stem cells were seeded onto CultiSphers, as described in the Materials and Methods, and cultured in spinner flasks for 1 week in mesenchymal stem cell growth medium. Stem cells on CultiSphers in one flask were cultured over the course of an additional 18 or 26 days in osteogenic supplement–containing medium (OS-positive) and a control flask was cultured using the medium (No OS). Samples were removed and alkaline phosphatase activity was determined. Each point is the average of three determinations ± SEM. There was no statistically significant difference ($p > 0.05$) between control samples (No OS) and OS-positive samples at day 18 or at day 26. However, there was a statistically significant difference (†$p < 0.01$) between day 18 and day 26 for both treatments.

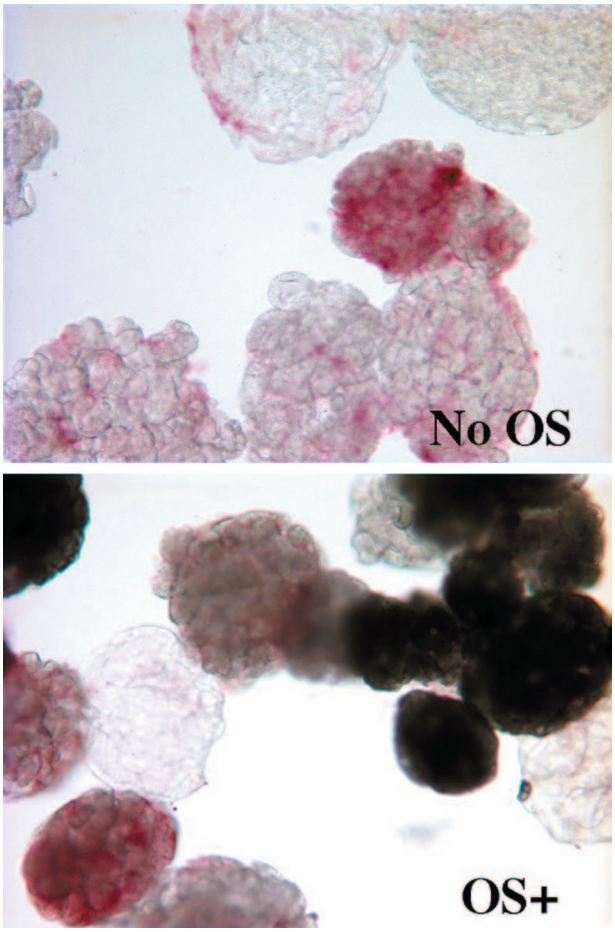

Fig. 6. Alkaline phosphatase activity of adipose-derived stem cells on CultiSphers after long-term culture shown by histochemical staining. Alkaline phosphatase–positive cells were detected on CultiSphers cultured in both control and osteogenic supplement medium. The distribution of alkaline phosphatase–positive cells was assessed by scoring individual CultiSphers using phase contrast microscopy. In osteogenic supplement medium, approximately 50 percent of the CultiSphers were opaque, representing deposition of a calcium-rich matrix, and could not be scored for alkaline phosphatase staining. No such opaque CultiSphers were observed in control cultures.

Table 3. Calcium Deposition by Adipose-Derived Stem Cells on CultiSphers*

	Day 18	Day 26
	(*ng calcium/CultiSpher*)	
No OS	0 ± 0	0 ± 0
OS-positive	760 ± 33†	2670 ± 89†

OS, osteogenic supplement.
*Adipose-derived stem cells were seeded onto CultiSphers, as described in the Materials and Methods, and cultured in spinner flasks for 1 week in mesenchymal stem cell growth medium. Stem cells on CultiSphers in one flask were cultured over the course of an additional 18 or 26 days in osteogenic supplement–containing medium (OS-positive), and a control flask was cultured using the growth medium (No OS). Samples were removed and calcium was determined. Each point is the average of three determinations ± SEM. Daggers indicate a statistically significant difference (†$p < 0.05$).

as acellular human dermis. All of these techniques have inherent problems. The autologous tissue operations are highly invasive and may involve significant donor-site morbidity. Implant reconstruction does not involve a donor wound, but is fraught with problems of capsular contracture and device failure over time. Biomaterials derived from mammals are subject to resorption, as are dermal and fat grafts. A regenerative strategy using a patient's own mesenchymal stem cells may represent the next evolutionary step in soft-tissue reconstruction. Adipose-derived stem cells can be easily isolated from suctioned fat and expanded in culture. They are better able to tolerate the mechanical trauma of the harvest techniques than mature fat cells. Moreover, these cells can be differentiated into mature fat cells in vitro using a defined inductive medium.

Adipose-derived stem cells also possess an osteogenic capacity.[11–16] Defined culture conditions will induce differentiation of these cells to osteoblasts with calcium deposition. Autologous bone grafting is a common procedure that is effective but associated with significant and prolonged pain at the donor site. In addition, harvest of bone graft from the extremities can be complicated by fractures. In contrast, harvest of adipose-derived stem cells using a standard liposuction cannula is performed with minimal discomfort and risk. Therefore, there is potential to stimulate stem cells toward osteogenic differentiation and implant them in a bony defect in lieu of autogenous bone.

One challenge to applying this cell-based regenerative strategy for bone and soft-tissue repair is finding an appropriate scaffold. Such a material should promote cell adhesion, proliferation, and differentiation, as well as prevent the implanted cells from migrating. Moreover, the scaffold material should not elicit a strong reaction from the host tissues. Macroporous collagenous beads provide a unique three-dimensional scaffold for cell attachment and proliferation in culture.[10–15] The small size of the beads allows adequate diffusion of nutrients throughout the structure while in culture and has provided a useful model for studying cell growth and contact in a three-dimensional environment.[2,17] Tissue-engineering applications for collagen beads have previously been explored, with the beads used to support mass culture of a hepatocyte cell line in the attempted construction of a bioartifical liver assist device.[13] Previous studies have also demonstrated that undifferentiated

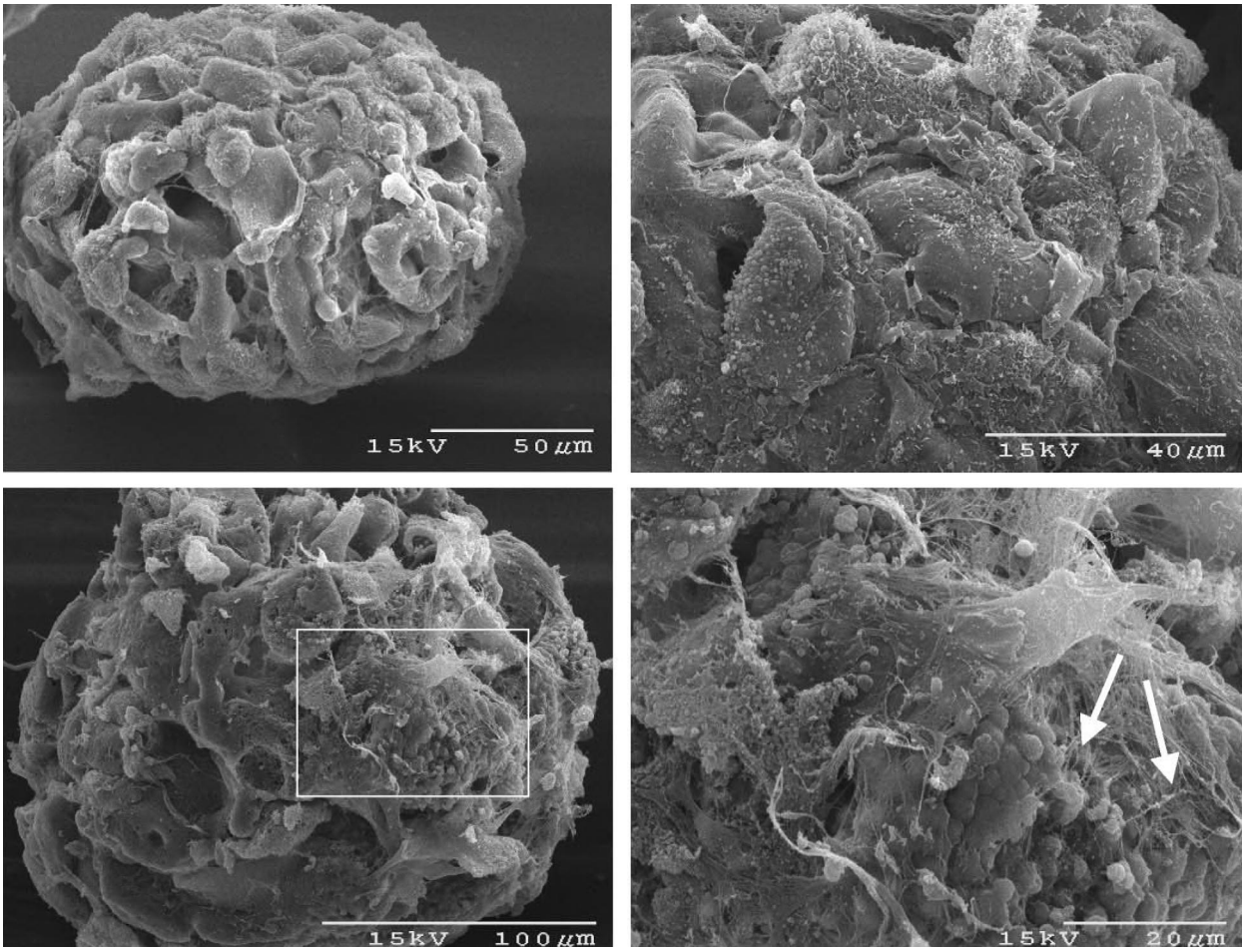

Fig. 7. Morphology of adipose-derived stem cells on CultiSphers after long-term culture using scanning electron microscopy. In both treatments, attached cells were readily detectable. In the osteogenic supplement–treated samples, structures characteristic of calcium phosphate deposition were observed (*arrows*). (*Above*) No osteogenic supplement; (*below*) osteogenic supplement–positive.

cell types can adhere to CultiSpher collagen beads in culture and be induced to differentiate to a desired phenotype.[11,15] Others have examined the proliferation and differentiation of adipose-derived stem cells on various carriers, such as agarose, alginate, and gelatin, and found that manipulating the composition of the scaffolds may have significant effects on their mechanical properties.[18] Furthermore, biodegradable polymer microspheres have been examined as an adipose-derived stem cell substrate with promising results.[19,20]

Collagen is especially applicable as a scaffold for clinical use because of its long track record as a Food and Drug Administration–approved injectable implant material. It is biodegradable within human tissues and has been used with minimal morbidity.[16] This study demonstrated that collagenous beads will support attachment and proliferation of adipose-derived stem cells as well as differentiation to osteogenic and adipogenic phenotypes. We have previously reported both the adipogenic and osteogenic potential of these stem cells in our laboratory.[21,22] The percentage differentiating to adipocytes when seeded on CultiSphers and exposed to adipogenic medium was observed to be lower than that for adipose-derived stem cells in cell culture plates. We speculate that the high degree of cell-to-cell contact in culture plates, a known factor in the promotion of adipogenesis, may lead to this difference. In contrast, adipose-derived stem cells were present at a low density on the CultiSphers when they were exposed to adipogenic medium.

Several recent studies have indicated the potential of adipose-derived stem cells to differentiate into osteoblasts. For example, Lecoeur and Ouhayoun[13] harvested extramedullary adipose tis-

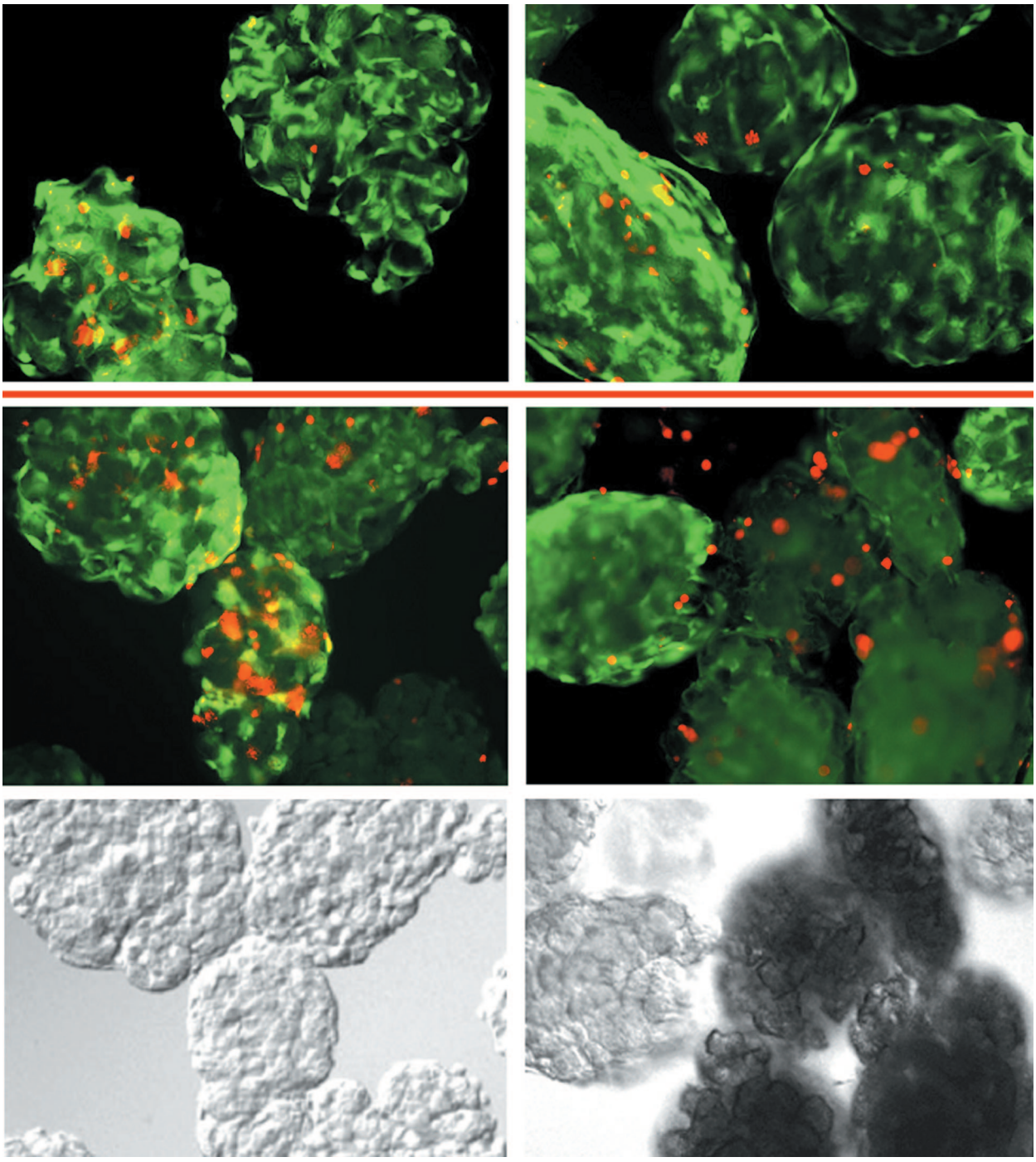

Fig. 8. Adipose-derived stem cells remain viable on CultiSphers after long-term culture. Samples were collected at 8 and 26 days, washed, and stained for viability using the Live (*green*)/Dead (*red*) fluorescence-based assay. Numerous viable cells were observed on CultiSphers cultured in both control (*left*) and osteogenic supplement (*right*) medium at both 8 days (*above*) and 26 days (*center* and *below*) of culture. After 26 days in osteogenic supplement medium, approximately 50 percent of the CultiSphers were opaque when viewed by Nomarski differential interference microscopy (*below*), indicating a calcium-rich matrix and resulting in less intense fluorescent staining.

sue from rabbits, treated the cells with bone morphogenetic protein (BMP)-2, and observed osteogenic differentiation. Lee et al.[14] demonstrated the differentiation of rat adipose-derived stem cells along osteogenic lineages in vitro using osteogenic supplement medium containing dexamethasone, β-glycerophosphate, and ascorbic acid, similar to the osteogenic supplement medium

used in our experiments. They reported in vivo bone formation from fat-derived stem cells after seeding cells in poly(glycolic acid) scaffolds and implanting them subcutaneously in a rat. Only the cells that differentiated into osteoblasts produced bone; the undifferentiated cells did not stain for osteocalcin. Dragoo et al. differentiated human stem cells derived from liposuction aspirates into osteoblasts by adding BMP-2 to the medium as well as by transfecting the stem cells with the BMP-2 gene.[11] This same group reported that implantation of human adipose-derived stem cells harvested from infrapatellar fat pads transfected with the BMP-2 gene also produced bone in the hindlimbs of nude mice.[12] Nathan et al. isolated adipose-derived, bone marrow–derived, and periosteum-derived stem cells from New Zealand White rabbits and implanted them (in a fibrin matrix) into a rabbit osteochondral defect.[15] Results indicated that cells were more readily obtained from adipose tissue than from bone marrow or periosteum and that gross osteochondral defect reconstitution and histological grading were superior to those in periosteum-derived stem cell repair. Recently, Gabbay et al. demonstrated the enhanced osteogenesis of adipose-derived stem cells in a three-dimensional matrix compared with a two-dimensional monolayer.[8]

Consistent with the reported ability of adipose-derived stem cells to differentiate into osteoblasts, those on CultiSphers cultured in the presence of an osteogenic supplement were able to deposit a calcium-rich matrix, as detected by chemical assay for calcium and scanning electron microscopy. We detected no calcium deposition by control cells. Assays for alkaline phosphatase activity, a commonly used marker for osteoblast differentiation, revealed statistically similar levels of activity in both control and osteogenic supplement–induced adipose-derived stem cells on CultiSphers.

CONCLUSIONS

We have demonstrated that a collagenous microbead carrier system is suitable for the expansion of human adipose-derived stem cells in culture and provides a favorable environment for adipogenic and osteoblastic differentiation. Cells adherent to the scaffolds showed a rapid doubling time, with extensive cell-to-cell contact. Importantly, differentiation of these stem cells to adipocytes and osteoblasts can be induced on this injectable matrix. Future studies will include in vivo studies to examine the potential of adipose-derived stem cells/CultiSpher constructs to form adipose tissue. The in vivo model will be a mouse subcutaneous injection, with an initial one-injection study followed by a study of multiple injections. We anticipate that this strategy may prove to be a useful therapy for tissue regeneration.

J. Peter Rubin, M.D.
Division of Plastic Surgery
University of Pittsburgh
682 Scaife Hall
3550 Terrace Street
Pittsburgh, Pa. 15261
rubinjp@upmc.edu

ACKNOWLEDGMENTS

The authors gratefully acknowledge Christa Heyward for electron micrographs, the Pittsburgh Tissue Engineering Technology Development Fund (J.S.D.), the McGowan Institute for Regenerative Medicine (K.G.M.), the Division of Plastic Surgery at the University of Pittsburgh (J.P.R. and K.G.M.), and National Institutes of Health grant RO1CA114246-01A1 (to J.P.R.).

DISCLOSURE

None of the authors has a financial interest in the products used in the experimental work.

REFERENCES

1. Zuk, P. A., Zhu, M., Ashjian, P., et al. Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell.* 13: 4279, 2002.
2. Zuk, P. A., Zhu, M., Mizuno, H., et al. Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng.* 7: 211, 2001.
3. Ashjian, P. H., Elbarbary, A. S., Edmonds, B., et al. In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. *Plast. Reconstr. Surg.* 111: 1922, 2003.
4. Safford, K. M., Hicok, K. C., Safford, S. D., et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem. Biophys. Res. Commun.* 294: 371, 2002.
5. Rangappa, S., Fen, C., Lee, E. H., Bongso, A., and Sim, E. K. Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. *Ann. Thorac. Surg.* 75: 775, 2004.
6. Cao, Y., Sun, A., Liao, L., Meng, Y., Han, Q., and Zhao, R. C. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. *Biochem. Biophys. Res. Commun.* 332: 370, 2005.
7. Rodriguez, L. V., Alfonso, Z., Zhang, R., Leung, J., Wu, B., and Ignarro, L. J. Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells *Proc. Natl. Acad. Sci. U.S.A.* 103: 12167, 2006.
8. Gabbay, J., Heller, J., Mitchell, S., et al. Osteogenic potentiation of human adipose-derived stem cells in a 3-dimensional matrix. *Ann. Plast. Surg.* 57: 89, 2006.
9. Ramirez-Zacarias, J. L., Castro-Munozledo, F., and Kuri-Harcuch, W. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* 97: 493, 1992.

10. Lowry, O. H., Roberts, N. R., Wu, M.-L., Hixson, W. S., and Crawford, E. J. The quantitative histochemistry of brain: Enzyme measurements. *J. Biol. Chem.* 207: 19, 1954.
11. Dragoo, J. L., Choi, J. Y., Lieberman, J. R., et al. Bone induction by BMP-2 transduced stem cells derived from human fat. *J. Orthop. Res.* 21: 622, 2003.
12. Dragoo, J. L., Samimi, B., Zhu, M., et al. Tissue-engineered cartilage and bone using stem cells from human infrapatellar fat pads. *J. Bone Joint Surg. (Br.)* 85: 740, 2003.
13. Lecoeur, L., and Ouhayoun, J. P. In vitro induction of osteogenic differentiation from non-osteogenic mesenchymal cells. *Biomaterials* 18: 989, 1997.
14. Lee, J. A., Parrett, B. M., Conejero, J. A., et al. Biological alchemy: Engineering bone and fat from fat-derived stem cells. *Ann. Plast. Surg.* 50: 610, 2003.
15. Nathan, S., DasDe, S., Thambyah, A., Fen, C., Goh, J., and Lee, E. H. Cell-based therapy in the repair of osteochondral defects: A novel use for adipose tissue. *Tissue Eng.* 9: 733, 2003.
16. Hosoya, M., Maruoka, Y., Oda, M., Asahina, I., Ichinose, S., and Omura, K. Bone with a vascular flap induced from fat tissue with the use of rhBMP-2 in rats. *J. Dent. Res.* 82: 581, 2003.
17. Rasey, J. S., Cornwell, M. M., Maurer, B. J., et al. Growth and radiation response of cells grown in macroporous gelatin microcarriers (CultiSpher-G). *Br. J. Cancer Suppl.* 27: S78, 1996.
18. Awad, H., Wickham, M., Leddy, H., Gimble, J., and Guilak, F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* 25: 3211, 2005.
19. Choi, Y., Cha, S., Lee, Y., Kwon, S., Park, C., and Kim, M. Adipogenic differentiation of adipose tissue derived adult stem cells in nude mouse. *Biochem. Biophys. Res. Commun.* 345: 631, 2006.
20. Choi, Y., Park, S., and Suh, H. Adipose tissue engineering using mesenchymal stem cells attached to injectable PLGA spheres. *Biomaterials* 26: 5855, 2005.
21. Clavijo, J. A., Rubin, J. P., Bennett, J. M., et al. A novel perfluoroelastomer with modified porosity enhanced adipose-derived stem cell survival in a mouse model. *Plast. Reconstr. Surg.* (in press).
22. Dudas, J. R., Marra, K. G., Cooper, G. M., et al. The osteogenic potential of adipose-derived stem cells for repair of rabbit calvarial defects. *Ann. Plast. Surg.* 56: 543, 2006.

Instructions for Authors: *Update*

Registering Clinical Trials

Beginning in July of 2007, *PRS* will require all articles reporting results of clinical trials to be registered in a public trials registry that is in conformity with the International Committee of Medical Journal Editors (ICMJE). All clinical trials, regardless of when they were completed, and secondary analyses of original clinical trials must be registered before submission of a manuscript based on the trial. Phase I trials designed to study pharmacokinetics or major toxicity are exempt.

Manuscripts reporting on clinical trials (as defined above) should indicate that the trial is registered and include the registry information on a separate page, immediately following the authors' financial disclosure information. Required registry information includes trial registry name, registration identification number, and the URL for the registry.

Trials should be registered in one of the following trial registries:

- http://www.clinicaltrials.gov/ (Clinical Trials)
- http://actr.org.au (Australian Clinical Trials Registry)
- http://isrctn.org (ISRCTN Register)
- http://www.trialregister.nl/trialreg/index.asp (Netherlands Trial Register)
- http://www.umin.ac.jp/ctr (UMIN Clinical Trials Registry)

More information on registering clinical trials can be found in the following article: Rohrich, R. J., and Longaker, M. T. Registering clinical trials in *Plastic and Reconstructive Surgery*. *Plast. Reconstr. Surg.* 119(3): 1097–1099, 2007.