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Angiogenic and Osteogenic Potential of Bone Repair Cells for Craniofacial Regeneration

Darnell Kaigler, D.D.S., M.S., Ph.D.,[1–3] Giorgio Pagni, D.D.S.,[1] Chan-Ho Park, M.S.,[1] Susan A. Tarle, B.S.,[1] Ronnda L. Bartel, Ph.D.,[4] and William V. Giannobile, D.D.S., DMSc[1–3]

There has been increased interest in the therapeutic potential of bone marrow derived cells for tissue engineering applications. Bone repair cells (BRCs) represent a unique cell population generated via an *ex vivo*, closed-system, automated cell expansion process, to drive the propagation of highly osteogenic and angiogenic cells for bone engineering applications. The aims of this study were (1) to evaluate the *in vitro* osteogenic and angiogenic potential of BRCs, and (2) to evaluate the bone and vascular regenerative potential of BRCs in a craniofacial clinical application. BRCs were produced from bone marrow aspirates and their phenotypes and multipotent potential characterized. Flow cytometry demonstrated that BRCs were enriched for mesenchymal and vascular phenotypes. Alkaline phosphatase and von Kossa staining were performed to assess osteogenic differentiation, and reverse transcriptase–polymerase chain reaction was used to determine the expression levels of bone specific factors. Angiogenic differentiation was determined through *in vitro* formation of tube-like structures and fluorescent labeling of endothelial cells. Finally, 6 weeks after BRC transplantation into a human jawbone defect, a biopsy of the regenerated site revealed highly vascularized, mineralized bone tissue formation. Taken together, these data provide evidence for the multilineage and clinical potential of BRCs for craniofacial regeneration.

Introduction

The demand for tissue replacements has led to the emergence and significant expansion of the field of tissue engineering.[1] Craniofacial regenerative medicine applications have demonstrated significant impacts for oral soft and hard tissue repair.[2,3] In preclinical model systems, multipotent cells derived from bone marrow have become a popular source of cells for regenerating bone, ligament, tendon, and cartilage.[4–8] More recently, autologous grafts utilizing various formulations of bone marrow or bone-marrow-derived cells have been investigated in clinical studies for skeletal bone repair[9,10] and craniofacial regeneration.[11–14] Although there has been modest success achieved in these approaches, major limitations still include crude isolation techniques and poorly defined cell preparations appropriate for grafting, inability to produce sufficient cell numbers for transplantation without multiple passages in traditional open tissue culture systems, and a lack of identification of an ideal cell type or cell population for transplantation. Despite the specific limitations of currently defined cell isolation and preparation protocols, an overarching challenge common to all cell and tissue transplantation strategies is the inability to produce a supportive vasculature for graft incorporation and cell survival.

It has been well-established that key to the development of bone tissue is not only the formation of bone but also the coordinated development of a supportive blood supply.[15] Thus, when employing cell transplantation strategies, not only does the osteoprogenitor cell type need to be considered, but formation of a functional vasculature to support cell viability and maturation of the tissue warrants serious consideration as well. As a result, some strategies used to engineer and regenerate bone tissue employ cotransplantation of osteoprogenitor cells with either hematopoietic or endothelial cells to help establish a supportive blood supply to the transplanted cells.[16–19] Although these approaches all hold great promise, it would be more desirable to transplant a cell population that contains cells capable of establishing both a blood supply and regenerating bone.

An automated cell-manufacturing process has been developed that utilizes a single-pass perfusion (SPP) process; this enables a clinical-scale expansion of autologous, primary, human bone repair cells (BRCs) derived from bone marrow. In SPP, the culture medium is continuously replaced with a fresh medium at a slow, controlled rate without the disturbance, removal, or passaging of cells. This technology results in significant expansion of primary human cells[20,21] and has previously demonstrated clinical

Departments of [1]Periodontics and Oral Medicine and [2]Biomedical Engineering, University of Michigan, Ann Arbor, Michigan.
[3]University of Michigan Center for Oral Health Research, Ann Arbor, Michigan.
[4]Aastrom Biosciences, Inc., Ann Arbor, Michigan.

success in the expansion of bone marrow and blood cells for replenishment of hematopoietic cells after treatment of various blood dyscrasias.[22–25] Additionally, results of recent *in vitro* and *in vivo* studies have generated interest in using this process to produce cells for bone tissue regeneration.[26]

The hypothesis underlying the current study is that BRCs have osteogenic and angiogenic potential that could manifest in their ability to regenerate bone and vascular tissue in a clinical craniofacial application. To address this hypothesis, we aimed to first examine the multipotency of BRCs *in vitro*, with particular emphasis on elucidation of their angiogenic and osteogenic capacities. Second, we sought to determine if BRCs could form bone and accelerate osteogenesis clinically when implanted within an osseous defect in the jaw.

Materials and Methods

Bone marrow harvest and BRC production

The production of BRCs has been previously described.[26] Briefly, after U.S. Food and Drug Administration (FDA) and University of Michigan Institutional Review Board approval, 10 healthy subjects giving informed consent underwent a bone marrow aspiration of the posterior ilium under conscious sedation and local anesthetic. Of these 10 subjects, where indicated, subsets of them (marrow) were used for phenotypic characterization of cells. Collected marrow was transferred to a sterile blood bag and bone marrow mononuclear cells (BMMNCs) were purified by Ficoll density gradient centrifugation. BMMNCs were then inoculated into a bioreactor, which is a proprietary computer-controlled, automated cell-processing unit, the Aastrom Replicell® System (Aastrom Biosciences). The Cell Cassette within this system provides a functionally closed, sterile environment in which cell production occurs. The fluid pathway in the Cell Cassette includes the cell growth chamber, a medium supply container, a mechanism for medium delivery, a waste medium collection container, and a container for the collection of harvested cells. All components are interconnected with sterile barrier elements throughout to protect the culture from contamination during use. The culture medium consists of Iscove's modified Dulbecco's medium, 10% fetal bovine serum, 10% horse serum, and 5 μM hydrocortisone. This system incorporates SPP in which a fresh medium flows slowly over cells without retention of waste metabolites or differentiating cytokines.[20] In-process safety test (absence of bacterial and fungal contaminants and endotoxins) was conducted on an sampled effluent medium at 48 h before harvest. After cultivation for 12 days at 37°C and 5% CO_2, the BRC product was harvested by trypsinization, washed in a physiologic buffer, and collected into a sterile bag and resuspended in Isolyte and 0.5% human serum albumin, where it was stored until the time of transplantation (Fig. 1). Sterility, endotoxin, and mycoplasma testing were all conducted on the BRCs.

Cell culture

The Replicell System is an automated one-cycle system that requires the input to be derived from fresh bone marrow aspirates; as a result, once BRCs are removed from the system, it is not possible to regrow or resume culture of cells within this same system. Thus, further culture of BRCs for

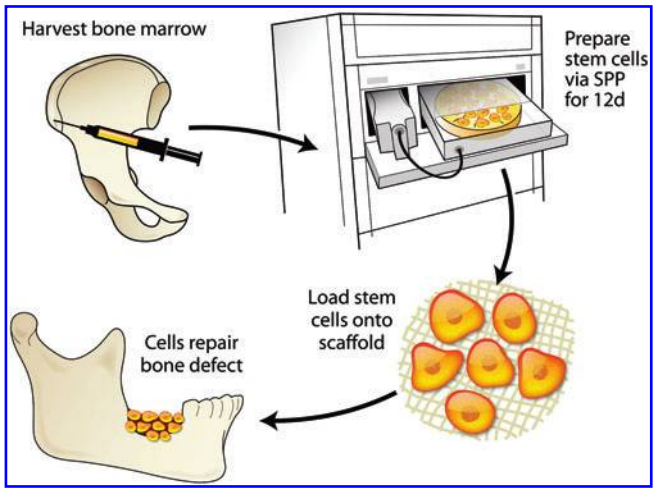

FIG. 1. Bone repair cell (BRC) production. After harvest of bone marrow aspirates, cells are cultured using an automated, closed-system, single-pass perfusion (SPP) process. After 12 days of cell expansion in this bioreactor system, cells are packaged and delivered to the bone regenerative site on a biodegradable sponge. Color images available online at www.liebertonline.com/ten.

in vitro assays required conventional tissue culturing techniques in both experimental and control conditions. Excess BRC product not utilized for transplantation was cultured in a medium consistent with culture of bone-marrow-derived stem cells,[27] consisting of minimum essential alpha medium (αMEM) (Gibco–Invitrogen #12571) with 15% fetal bovine serum (Gibco-Invitrogen-16000), 100 μM ascorbic acid 2 phosphate (Sigma A-8960), and 5 μg/mL Gentamicin (Invitrogen #15750060), and grown in a 37°C humidified tissue culture incubator under 5% CO_2. This medium was used for control conditions (without incorporation of differentiation/inductive factors) in the *in vitro* assays and it should also be noted that this open culture system and media used were distinctly different from those used to produce the BRC product. Cell culture media were changed every 2 or 3 days. Cells were grown in T-150 flasks to ~80% confluence, followed by cell washing with phosphate-buffered saline (PBS) and trypsinization before being split into 12-well plates or 60 mm tissue culture dishes for differentiation assays.

Flow cytometry

Phenotypic comparison of cell surface marker expression was made between the starting BMMNCs and the final BRC product from a subset of four subjects who had marrow harvested for generation of BRCs. Flow cytometry was performed on the same day of isolation of the BMMNCs and immediately after production of BRCs (i.e., neither BMMNCs nor BRCs were cultured, as described in the Cell Culture section, before performance of the flow cytometry). BMMNCs or BRCs were washed and resuspended in 1× Dulbecco's PBS (Gibco) containing 1% bovine serum albumin. Samples were F_c receptor blocked with normal mouse IgG for approximately 10 min. Tubes containing 0.5×10^6 cells in approximately 0.1 mL were then stained at 2°C–8°C with phycoerythrin (PE) or PE cyanin 5-conjugated anti-CD90 (Thy1) antibodies, PE-conjugated anti-CD11b, anti-Gly-A, anti-CD34, anti-AC133, anti-CD19, anti-vascular endothelial

growth factor (VEGF)R1, anti-Tie2, anti-CD145, fluourescein isothyocyanate-conjugated anti-CD66b, anti-CD14, anti-CD45, anti-CD3, anti-CD144 antibodies, and PE cyanin 5-conjugated anti-CXCR4 (CD184) (Beckman Coulter). After 15 min, cells were washed and resuspended in 0.5 mL PBS/bovine serum albumin for analysis on the Epics XL-MCL (Beckman Coulter) or FC500 flow cytometer.

In vitro *multilineage differentiation*

Multipotency of BRC cultures was determined by *in vitro* differentiation assays of osteogenesis, chondrogenesis, and adipogenesis. BRCs were plated at a density of 30,000 cells per well in 12-well plates. At >90% confluency, cells were induced, according to previously described protocols,[28] with osteogenic (BRC medium including 5 mM β-glycerol phosphate, 100 nM dexamethasone, and 50 μM ascorbic acid 2-phosphate), chondrogenic (BRC medium including 50 μM ascorbic acid 2-phosphate, 100 nM dexamethasone, 5 μg/mL human insulin, 1 ng/mL transforming growth factor-β, 400 μM proline, and 1× Nonessential amino acids), or adipogenic (BRC medium including 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μg/mL human insulin, and 200 μM indomethacin) medium. BRCs were grown at 37°C in a humidified 5% CO_2 incubator. The medium was changed every 2–3 days. After 2 weeks, the cells were fixed and stained as outlined below.

Mesenchymal differentiation staining

To detect chondrogenic differentiation, induced BRCs were fixed in cold 100% methanol for 30 min and then exposed to 1% Alcian blue in 0.1 N HCl for 30 min. Cells were washed twice with 0.1 N HCl. Cells in PBS were viewed or stored at 4°C.

To detect mineralized matrix formation indicative of osteogenic differentiation, induced BRCs were fixed in 4% paraformaldehyde for 30 min, immersed in fresh 5% silver nitrate, and incubated in the dark for 30 min. After washing in water, the BRCs were exposed to ultraviolet light for 30 min followed by a 4-min incubation in 1% sodium thiosulfate to neutralize the silver nitrate. Cells were washed and stored in PBS at 4°C.

To detect adipogenic differentiation by identifying lipid vesicles, induced BRCs were fixed in 4% paraformaldehyde for 30 min and then immersed in 0.3% Oil Red O solution (in isopropanol) for 30 min. Cells were washed and stored in PBS at 4°C.

Alkaline phosphatase activity and detection

Early osteogenic differentiation was detected and quantified by alkaline phosphatase (ALP) staining and ALP enzyme assays. ALP activity was confirmed with reverse transcriptase–polymerase chain reaction (PCR). Cells were plated at a density of 30,000 cells per well in 12-well plates. At 80% confluence, cells were induced with the osteogenic medium as described above. The medium was changed every 2–3 days and after 1 week, and ALP activity was measured.

To detect ALP activity, the BRCs were fixed in 70% ethanol for 30 min. They were then incubated with freshly made substrate containing naphthol AS-TR phosphate (Sigma) and Fast blue for 30 min. Cells were washed twice with PBS and then viewed or stored at 4°C.

To quantify the ALP activity and normalize the results, BRCs were lysed in Passive Lysis Buffer (Promega) according to manufacturer's instructions. Cell lysates were then sonicated, and centrifuged (10,000 rpm for 10 min at 4°C). The supernatant was recovered for the quantitative colormetric ALP assay,[29] and the cell pellet was used for DNA isolation and the determination of the DNA concentration using the Quant-iT™ dsDNA BR Assay (Invitrogen) per the manufacturer's instructions.

Reverse transcriptase–PCR

To further confirm osteogenic differentiation, after 1 week of osteogenic induction total BRC cellular RNA was extracted, reverse transcribed, and amplified using osteoblast-specific gene primers: *Runx2*, osteocalcin (*OCN*), and bone sialoprotein (*BSP*). Media from the wells of induced BRCs were aspirated. Cells were immediately resuspended in 1 mL of Trizol (Invitrogen) and RNA was isolated according to the manufacturer's instructions. cDNA was synthesized using Invitrogen's SuperScriptII kit and oligo dT. PCR reaction components and concentrations were as described in the Invitrogen Platinum Taq polymerase instructions using the primer sets below. An MJ themorcycler (MJ Resarch Model PTC-200) was used for the two PCR reaction conditions (Table 1).

Angiogenic potential

The angiogenic potential of BRCs was investigated through the fluorescent labeling of endothelial cells and capillary tube formation in culture. Collagen culture dishes were prepared by dispensing 2.5 mL of an ice-cold collagen

Table 1. Polymerase Chain Reaction Primer Pairs

Primer name	*Primer sequence*	*Product size (bp)*	*Accession number*
BSP BSP FWD[b]	CTATGGAGAGGACGCCACGCCTGG	586	NM_004967
BSP REV[b]	CATAGCCATCGTAGCCTTGTCCT		
GAPDH FWD[a]	AGCCGCATCTTCTTTTGCGTC	815	NM_002046
GAPDH REV[a]	TCATATTTGGCAGGTTTTTCT		
OCN FWD[a]	CATGAGAGCCCTCACA	314	NM_199173
OCN REV[a]	AGAGCGACACCCTAGAC		
Runx2 FWD[b]	CCCGTGGCCTTCAAGGT	76	NM_004348
Runx2 REV[b]	CGTTACCCGCCATGACAGTA		

[a]94°C 2 min [94°C 45″ 56°C 45″ 72°C 1′] ×35 cycles 72°C 15′.
[b]94°C 2 min [94°C 45″ 67°C 45″ 72°C 1′] ×35 cycles 72°C 15′.

solution, containing 2.4 mg/mL PureCol® bovine collagen (Advanced BioMatrix) in PBS at pH 7.4 into 60 mm plates. Collagen plates were solidified at 37°C for 90 min and then equilibrated in the growth medium for 2 h. BRC cultures were plated with 200,000 BRCs and incubated overnight at 37°C. One day after plating the cells, the medium was changed with a medium containing 50 ng/mL VEGF (R&D #293-VE). The medium was changed every 2–3 days. After 1 week, cells were fed the medium containing 10 μg/mL fluorescently labeled lipoprotein (DiI-Ac-LDL; Biomedical Technologies) for 4 h at 37°C to specifically label the endothelial cells. The medium was aspirated and the cells were washed three times with PBS before being fixed in 4% paraformaldehyde for 60 min. After fixing, the cells were washed three times with water before 3 mL of PBS was added to each plate, and then viewed or stored at 4°C.

To measure angiogenic cytokine production, BRCs from a subset of eight subjects were centrifuged to pellet the cells, and resuspended in X-Vivo 15 (Lonza) medium. X-Vivo 15 is a serum-free medium that was used in these assays to eliminate potential serum interference and nonspecific binding with some of the reagents (i.e., lack of human specific antibodies) used in the assays. All fractions were quantified and diluted to 1×10^6 mL^{-1} in X-VIVO 15 medium. The cells (100 μL) were added to the wells of a 96-well round-bottom plate in triplicate. The culture medium alone or supplemented with 300 ng/mL endotoxin lipopolysaccharide was added in a volume of 50 μL to all wells. Twent-four hours later, 100 μL of supernatant from each well was collected and stored at −70°C until analysis. Human Cytokine/Chemokine LINCOplex kits (Millipore Corporation) were used for multiplex analysis of VEGF and IL-8 concentrations in BRC supernatant fluids. The kits were used for cytokine determination as defined by the manufacturer's protocol (HCYTO-60K-Rev. 12/07/05) located at the following link:

www.millipore.com/userguides.nsf/a73664f9f981af8c852569b9005b4eee/09dc59783f6430ab85257259004f0203/$FILE/hcyto-60k.pdf.

ELISA kits (R&D Systems) were used to quantify the individual analytes Tie-1 and Tie-2 according to the manufacturer's protocol.

Surgical transplantation of BRCs to a localized craniofacial osseous defect

A nonrestorable tooth was extracted under local anesthesia from a patient in need of tooth extraction due to dental decay and periodontal disease associated with the tooth. Due to the preexisting condition of the tooth, its removal resulted in an area of a localized bone defect. According to a protocol approved by the U.S. FDA and the University of Michigan's Institutional Review Board, a full-thickness mucoperiosteal flap was elevated for access and hemostasis of the surgical defect achieved. One milliliter of the BRC cell suspension (approximately 1.5×10^7 cells/mL) was absorbed onto a gelatin sponge (Gelfoam; Pfizer) sized to a dimension of $\sim2\,cm^3$. The sponge was then transplanted into the extraction site to the level of the interproximal bone. The flap was then coronally repositioned, and a bioabsorbable collagen barrier membrane (Biomend®; Zimmer Dental) was placed over the sponge to contain the cell construct. The tissues were approximated and closed with a 4-0 bioabsorbable suture material. The subject was prescribed 500 mg amoxicillin, three times a day (t.i.d.) for 7 days, decreasing doses of dexamethasone (8, 4, 2, 2 mg) for 4 days, and 600 mg Ibuprofen four times a day (q.i.d.) for 3 days. Surgical reentry of the treated osseous defect was performed 6 weeks postsurgery, and a bone core of 2×7 mm was harvested with a trephine drill (Ace Surgical Supply Co., Inc.). To ensure harvesting of bone from the area where the BRCs were grafted, pre- and postsurgical measurement templates were used to identify the area corresponding to the center of the previous extraction socket. The core was prepared for microcomputed tomography (μCT) imaging and descriptive histological evaluation.

μCT and histology

The nondecalcified core was captured with the scanning direction parallel to the longitudinal axis of the core specimen. High-resolution scanning with an in-plane pixel size and slice thickness of 24 μm was performed. To cover the entire thickness of the bone core biopsy, the number of slices was set at 400. GEMS MicroView® software was used to make three-dimensional (3D) reconstructions from the set of scans as previously described.[30]

After scanning, the core was decalcified for 2 weeks in 10% ethylenediaminetetraacetic acid. After decalcification, the core was transferred to 70% ethanol stored at 4°C until ready for embedding in paraffin. Standard hematoxylin and eosin staining was performed to assess bone morphology and blood vessel formation.

Statistical analysis

Statistical analysis was performed with the use of Instat software (GraphPad Software). All data were plotted as mean ± standard error of the mean, unless otherwise noted. Statistically significant differences were determined by two-tailed Student's *t*-tests, and statistical significance was defined as $p < 0.05$.

Results

Phenotypic characterization of BRC population

For all BRC formulations produced after cell processing, flow cytometry was performed to characterize the phenotype of the BRCs, and in some instances, the initial BMMNCs were also assessed before production of BRCs. For 10 subjects from whom BRCs were produced, including the subject treated with BRCs in this study, cell surface marker expression of the final BRC product is shown (Table 2). There are high percentages of CD31+, CD90+ (Thy1), and CD105+ cells. When compared to cell surface marker expression from typical BMMNC fractions used to produce these cells (unpublished proprietary data), CD90+ (Thy1) and CD105+ are highly enriched in the BRC product.

Mesenchymal potential of BRCs

BRCs were assessed for their capacity to differentiate toward different cellular lineages after culture under adipogenic, chondrogenic, and osteogenic conditions. After 2 weeks of culture, BRCs were subjected to Oil Red O, Alcian

Table 2. Frequency of Cell Phenotypes in Bone Repair Cells ($N = 10$) Produced by Single-Pass Perfusion (Mean ± Standard Deviation)

Phenotype	*BRC*	*SD*
% Viable (% 7AAD−)	92.69	2.16
% CD11b+	61.17	5.70
% CD66b+	28.20	7.50
% Lin-CD34+	0.61	0.35
% CD133+	0.38	0.13
% VEGFR2	6.15	2.39
% CD14 + Auto−	10.57	2.57
% CD14 + Auto+	16.81	6.89
% CD45+	79.24	3.39
% CD3+	14.24	5.41
% CD19+	0.81	0.47
% CD31+	65.61	5.69
% CD90 (Thy-1)+	17.00	2.85
% CD105+	20.20	1.85
% CD105 + CD90+	15.79	2.98
% CD90 + s that are 105+	94.44	3.05

BRC, bone repair cell; SD, standard deviation; VEGF, vascular endothelial growth factor.

blue, and von Kossa staining. In control culture conditions, Oil Red O, Alcian blue, and von Kossa staining were all negative. In adipogenic conditions, Oil Red O staining was used to detect intracellular lipid-rich vacuoles and morphological changes in cell shape. The results confirmed that cells were differentiated toward an adipogenic lineage (Fig. 2a). Similarly, in cells grown under chondrogenic conditions, the presence of chondrogenic proteoglycans was indicated by positive Alcian blue staining (Fig. 2a), confirming chondrogenic differentiation. Under osteogenic culture conditions, deposition of mineralized matrix indicative of osteoblasts was evident through positive von Kossa staining (Fig. 2a). To further evaluate osteogenic differentiation, alkaline phophatase activity was also analyzed qualitatively and quantitatively after 1 week of culture in osteogenic conditions (Fig. 2b). With ALP staining, there was a significantly more robust and widespread degree of staining in the osteogenic conditions relative to the control culture conditions. When this difference was measured quantitatively, there was a significant threefold increase ($p < 0.05$) in ALP activity in osteogenic relative to control conditions. Commitment toward an osteogenic lineage was further confirmed through gene expression of *Runx2*, *OCN*, and *BSP* (Fig. 2c), and although *Runx2* was expressed at a lower level in the control condition (data not shown), *OCN* and *BSP* were not detectable in control conditions at this timepoint.

Angiogenic potential of BRCs

In addition to standard phenotypic marker characterization of the final BRC product (shown from 10 patients treated with BRCs in Table 2), further analysis was performed on BRC products produced from smaller subsets of healthy volunteer subjects who provided donor marrow specifically to evaluate marker expression of angiogenic phenotypes. First, phenotypic comparison of cell surface marker expression was made between the starting BMMNCs and the final BRC product from a subset of four subjects. After expansion of the BMMNCs using the BRC process, the enrichment of vascular phenotypes in BRCs is shown in Table 3. Cell surface expression of vascular phenotypes associated with endothelial cells (Angiopoietin and flt1) and pericytes (Thy1+ and MUC-18) are all highly enriched. Next, when BRCs from a subset of eight volunteer donors (from the 10) were cultured in a medium (X-VIVO) without addition of VEGF, cells were shown to produce appreciable levels of potent angiogenic cytokines VEGF, and angiopoietin (Ang)-1 and -2 (Fig. 3a). It should be noted that none of these cytokines were detected in the supernatant from the initial BMMNCs used to produce BRCs. Another angiogenic cytokine, interleukin-8 (Il-8), was produced by BRCs at a concentration (7.5 ng/mL/100,000 cells) significantly higher than that in the initial BMMNCs (4.6 ng/mL/100,000).

To examine the differentiation of BRCs into endothelial and vascular cells, BRCs were cultured in 3D collagen gels in the presence of VEGF. In these assays, VEGF is typically incorporated to induce endothelial cell differentiation.[31–33] Over the course of 1 week, cells elongated and formed spindle-like interconnecting structures resembling those seen by endothelial cells forming capillary tubes when grown under similar culture conditions (i.e., 3D extracellular matrices) (Fig. 3b). To further confirm the differentiation of BRCs into endothelial cells, a fluorescently labeled lipoprotein (DiI-Ac-LDL) that is metabolized specifically by endothelial cells was added to BRC 3D cultures. After 4 h of incubation with DiI-Ac-LDL, cells were examined under fluorescence microscopy and demonstrated uptake of the fluorescently labeled dye (Fig. 3c, d), characteristically seen by endothelial cells.[34] Taken together, these data demonstrate that BRCs have the capacity to differentiate into endothelial cells in 3D extracellular matrices and produce soluble angiogenic factors.

Clinical osteogenic and vascular regenerative potential of BRCs

The ultimate test of the osteogenic and angiogenic potential of BRCs is their ability to regenerate bone and vascular structures in a clinical model. To address this clinical situation, we grafted BRCs into an osseous defect of the jawbone. After tooth extraction, a bone defect resulted in the area that the tooth previously occupied. The BRCs (in liquid suspension) were carefully placed (Fig. 4a) onto a resorbable material matrix (gelatin sponge) to the point of saturation (Fig. 4b). Cells were allowed 15 min to adhere to the gelatin sponge before their placement into the recipient graft site. After the extent of the bone defect (created as a result of tooth removal) was assessed (Fig. 4c), the sponge matrix containing the BRCs was grafted into the bone defect (Fig. 4d), a collagen membrane was placed for containment of the graft (Fig. 4e, f), and the area then closed with sutures and allowed to heal for 6 weeks.

After 6 weeks of healing, the grafted defect site was reentered for examination of regenerated tissue and dental implant placement. Clinical examination revealed the appearance of bone tissue, with no evidence of the previous bone defect (Fig. 5a), and a core biopsy was harvested from the center of the area in which the BRCs were grafted. Upon retrieval of the specimen with the trephine drill, it was found that the tissue was very dense, indicative of mature bone

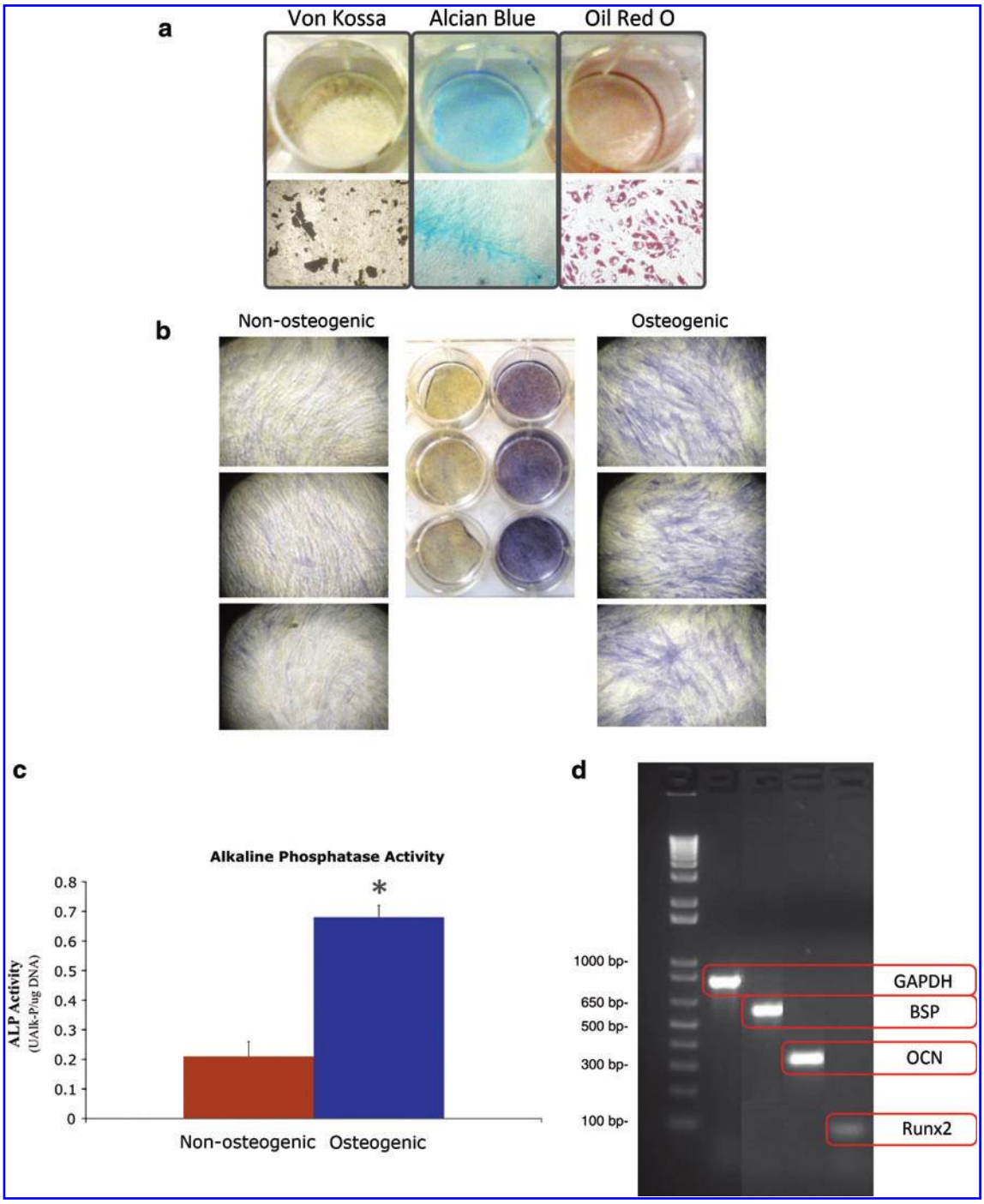

FIG. 2. Multipotent and osteogenic potential of BRCs. (**a**) After induction of BRCs in osteogenic, chondrogenic, and adipogenic media, photographs and corresponding photomicrographs show multipotent mesenchymal differentiation as measured by phenotypic expression of osteogenic mineralized matrix (von Kossa), chondrogenic proteoglycans (Alcian blue), and adipogenic lipid vacuoles (Oil Red O); low magnification images were taken at 40×; high magnification images are shown at 200×. (**b**) Osteogenic induction potential of BRCs is further evaluated qualitatively (staining) and (**c**) Quantitatively through measurement of alkaline phosphatase (ALP) activity of cells grown in the control medium versus cells grown in the osteogenic medium (*$p < 0.05$; alkaline phosphatase assays were performed in triplicate, and high-magnification non-osteogenic and osteogenic panels shown on the ends represent the same wells shown at lower magnification in middle panel); low magnification images were taken at 40×; high magnification images are shown at 200×. (**d**) BRC expression of bone-specific transcripts (*Runx2*, osteocalcin [*OCN*], and bone sialoprotein [*BSP*]) was also measured with reverse transcriptase–polymerase chain reaction. Color images available online at www.liebertonline.com/ten.

Table 3. Presence and Enrichment of Vascular Phenotypes in Bone Marrow Mononuclear Fraction and Bone Repair Cell Product

Phenotype	*Common name*	*Cell type recognized*	*BMMNC (mean ± SD)*	*BRC (mean ± SD)*	*Fold enrichment*
CD34+	—	HSCs/ECs progenitors	3.5 + 1.2	0.6 + 0.3	<1
CD133+	AC133	HSCs/ECs progenitors	1.2 + 0.2	0.5 + 0.2	<1
CD90+	Thy1	SSCs/pericytes	0.3 + 0.2	28.2 + 10.7	107.5
CD202b+	Tie2/Angiopoietin receptor	HSCs/ECs	7.3 + 4.8	35.7 + 6.3	4.9
—	VEGFR1/flt 1	Hematopoietic	6.5 + 2.0	38.9 + 4.7	6.0
CD144+	VE-Cadherin	Vascular ECs	2.5 + 2.4	0.9 + 0.6	<1
CD146+	MUC18, S-endo	ECs/pericytes	1.2 + 0.5	27.1 + 8.5	23.4

Values represent means taken from four different subjects. BMMNC, bone marrow mononuclear cell; HSCs, hematopoietic stem cells; ECs, endothelial cells; SSCs, skeletal (mesenchymal) stem cells.

tissue (Fig. 5b). The clinical appearance of the biopsy specimen clearly showed a highly vascular tissue that, consistent with retrieval of viable bone tissue, produced bleeding after harvest (Fig. 5c). μCT analysis was performed on the biopsied tissue, and 3D reconstruction of the specimen showed highly mineralized tissue throughout the entire length of the core (Fig. 5d). After this analysis, histology was performed on the specimen and hematoxylin and eosin staining clearly showed widespread distribution of mature bone tissue with an abundance of blood vessels distributed throughout

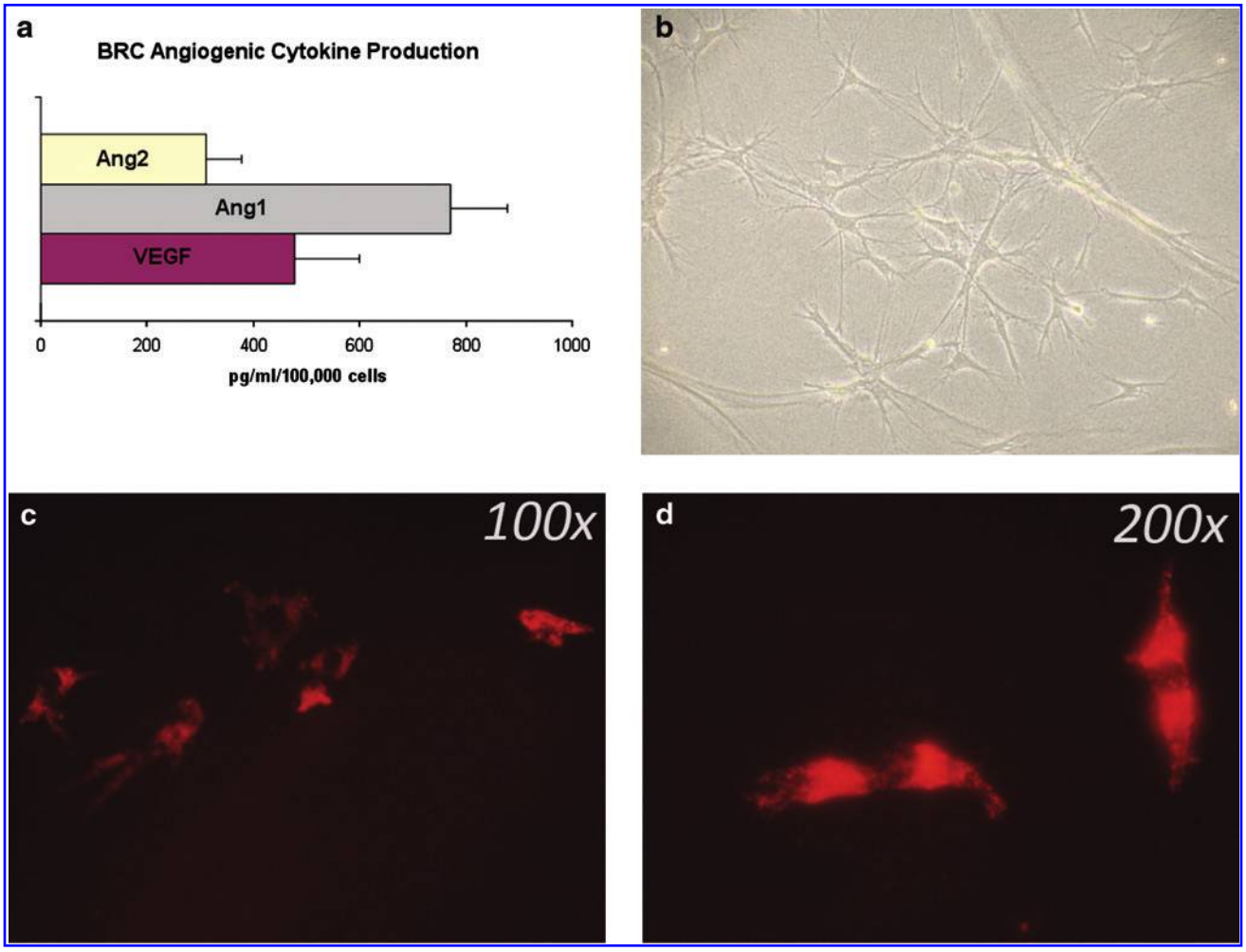

FIG. 3. *In vitro* angiogenic phenotype of BRCs. (**a**) BRCs ($n = 8$) were shown to produce clinically appreciable levels of potent angiogenic cytokines, vascular endothelial growth factor (VEGF), and angiopoietin (Ang)-1 and -2. (**b**) After BRCs were cultured over 5 days in the presence of 50 ng/mL VEGF within three-dimensional collagen extracellular matrices, photomicrographs were taken and show sprouting structures indicative of endothelial cell capillary tube formation (100× magnification). (**c**) 100× and (**d**) 200× photomicrographs of fluorescently labeled BRCs after 4-h incubation with a (w/a) fluorescent-labeled lipoprotein (DiI-Ac-LDL) metabolized specifically by endothelial cells. Color images available online at www.liebertonline.com/ten.

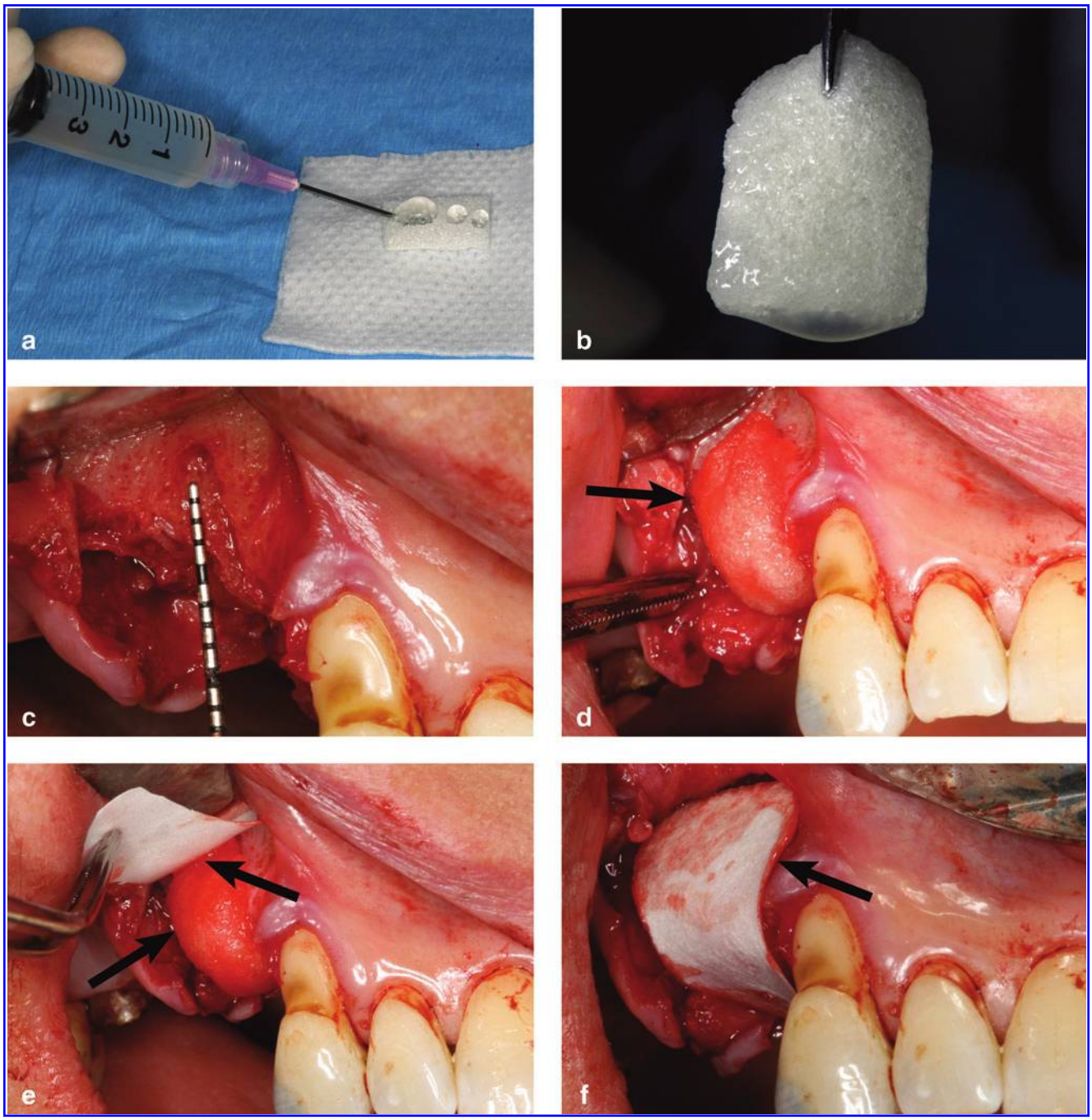

FIG. 4. Grafting of BRCs into localized jawbone defects. (**a**) Initial placement of BRCs onto gelatin sponge and (**b**) saturation of sponge with BRCs just before implantation into jawbone defect. Photographs of (**c**) jawbone defect after tooth removal, (**d**) placement of BRC-loaded gelatin into jawbone defect (arrows point to cell loaded gelatin), and (**e, f**) placement of a protective collagen membrane (arrows) for graft containment. Color images available online at www.liebertonline.com/ten.

(Fig. 5e). These results provide preliminary evidence that BRCs, produced from BMMNCs, have the regenerative capacity to produce highly vascular bone tissue in a human craniofacial bone defect.

Discussion

Cell transplantation of stem cells has tremendous potential for craniofacial regenerative applications; yet, identification of the appropriate cell types and cell processing protocols are two of the most critical determinants in producing successful outcomes. In the present study, our aim was to assess the capacity of a cell production process, which utilizes an automated closed-system bioreactor, to produce clinical-scale numbers of autologous cells (BRCs) capable of regenerating clinically viable bone. Through cell surface marker characterization of BRCs, it was determined that the SPP *ex vivo* cell expansion processing of bone marrow aspirate was capable

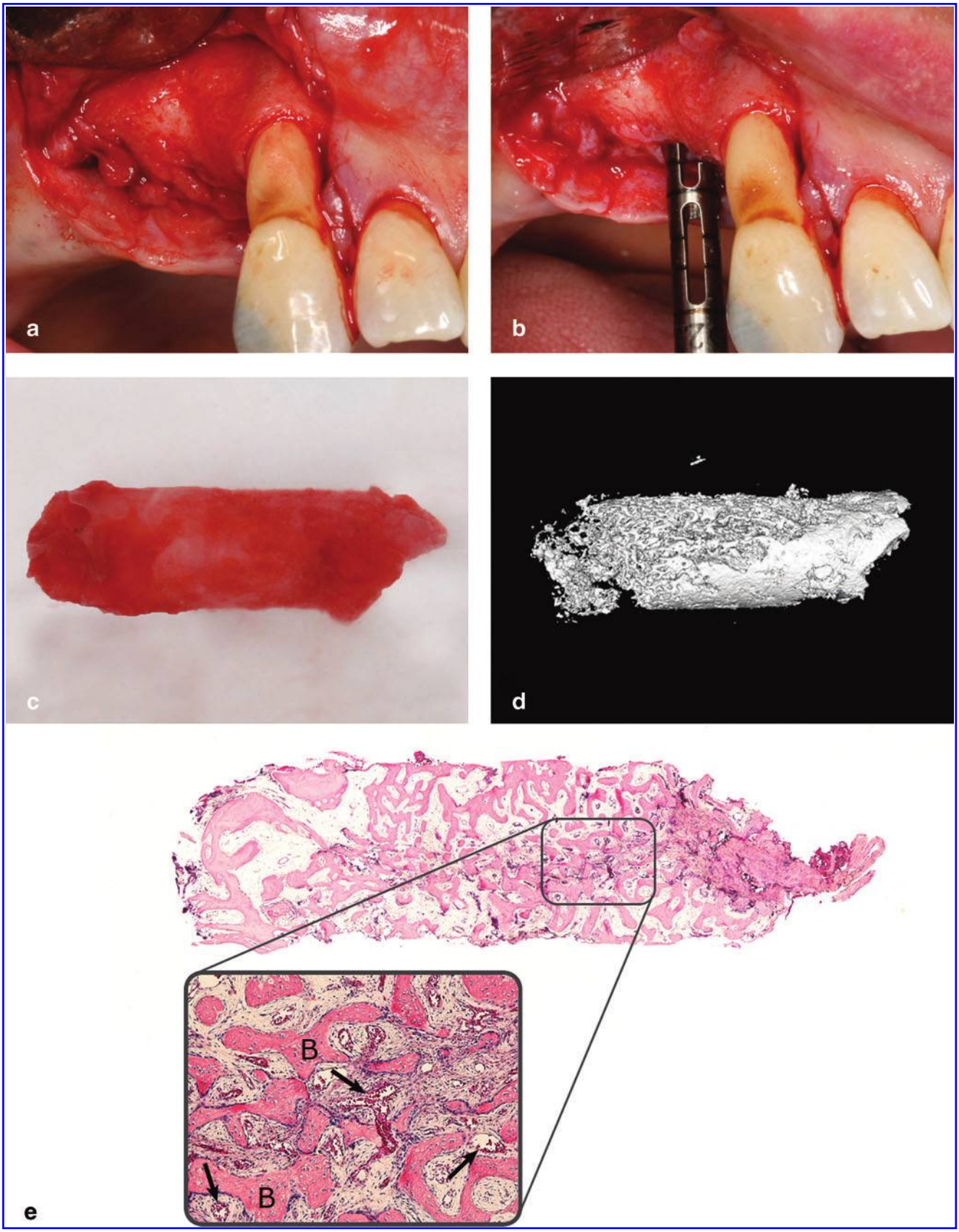

FIG. 5. BRC regeneration of highly vascular bone in jawbone defect. Photomicrographs of (**a**) regenerated jawbone defect 6 weeks after grafting of BRCs, (**b**) harvesting of bone core biopsy using trephine bur (just before placement of dental implant), and (**c**) bone core biopsy. (**d**) Microcomputed tomography three-dimensional reconstruction of bone core biopsy from BRC-regenerated bone demonstrating mineralized tissue formation throughout specimen. (**e**) Histological evaluation (hematoxylin and eosin staining) of bone formation showing areas of mature cortical bone (B) with high vascularity, as indicated by abundance of blood vessels (arrows; low magnification at 4× and high magnification at 200×). Color images available online at www.liebertonline.com/ten.

of producing cell populations highly enriched for mesenchymal and vascular progenitor cells. This was confirmed through cell characterization of BRCs, where cells within this heterogeneous population demonstrated the capacity to be induced to differentiate down chondrogenic, adipogenic, osteogenic, and angiogenic lineages. Finally, the test of the clinical regenerative capacity of BRCs demonstrated their ability to regenerate highly vascular bone in a human jawbone defect.

The cell-processing system employed to produce these cells utilizes an SPP protocol.[20,22,25,35] Gastens *et al*.[35] examined this system and its ability to expand bone-marrow-derived cells to produce clinical-scale cell numbers. These initial studies compared cell phenotypes of cells produced with this closed SPP system to phenotypes of cells cultured with an open system utilizing conventional tissue culture parameters. Although mesenchymal cell numbers were observed to be higher in cells cultured with the SPP closed system, the limitation of the study was that the initial BMMNC fractions tested in the two different systems came from different donors; thus, differences observed between the final cell products could have been, at least in part, attributed to donor–donor variability. In the present study, although we did not compare cell populations from closed and open culture systems, we did compare BRCs directly to the phenotypes of the BMMNCs from which they were produced. The final BRC population showed marked enrichment for mesenchymal and vascular cell phenotypes, suggesting that for therapeutic regenerative strategies, the SPP process supports the production of a more favorable cell population for transplantation than protocols using transplants comprised of unfractioned, whole bone marrow. Although there is often a wide degree of variability in the phenotypic expression of these markers from donor to donor, the relative differences in cell phenotype before and after cell processing is consistent in that the final product is enriched in expression of mesenchymal and angiogenic phenotypes after cell processing with the SPP system. Additional studies need to be performed comparing cell phenotypes and differentiation potential of cells derived from the same donor, when processed with either this closed system or a traditional tissue culture open system technique. It is also important to note that although the objectives of the differentiation assays were to examine the osteogenic and angiogenic potential of the BRCs, it is not possible to regrow or resume culture of cells in the Replicell system once they have been removed from the system. As such, further culture of BRCs required conventional tissue culturing techniques. However, it is recognized that this additional culture step could have potentially resulted in a cell population not identical to the population produced from the Replicell system; yet, because the Replicell system is a closed system, there is not a viable alternative to performing or adapting the aforementioned assays to cells cultured while in the Replicell system.

The ability of the SPP process to form bone-forming cells has also been recently studied *in vitro* and in ectopic animal models.[26] In these studies, the levels of bone formation seen *in vivo* followed the same trends of the osteogenic differentiation observed *in vitro*. Additionally, cell surface markers, including CD90+ (Thy1) and CD105+ (endoglin), were positively correlated with ectopic bone formation in mice. CD105, originally identified as a marker of mesenchymal stem cells,[36] has more recently been associated with vascular endothelium in angiogenic tissues[37] and expression of CD90 has been linked to bone marrow subpopulations of colony-forming mesenchymal stem cells (CFU-F, colony-forming unit–fibroblasts).[38] In our study, cell surface marker expression of BRCs from the subject who underwent cell transplantation in the bone defect showed 65-fold and 5-fold increases in CD90+ and CD105+, respectively (data not shown). This served as an indication that the BRC product was highly enriched with cells possessing mesenchymal and angiogenic potential and is in accordance with previous reports demonstrating enrichment of theses cell types with this cell-processing protocol.[26,35]

Although the cell product is highly enriched for vascular and mesenchymal cells as indicated by cell surface marker expression and *in vitro* differentiation capacity, it is clear that *in vitro* osteogenic differentiation of cell populations does not guarantee bone-forming capacity *in vivo*. Meijer *et al*. performed a clinical study evaluating the repair of jawbone defects in six subjects treated with autologous cells expanded using an open system,[39] similar to the protocol used in traditional tissue culture of mesencyhmal stem cells.[40,41] In this study, cells were cultured anywhere from 12 to 25 days before implantation and the last 7 days in culture cells were grown on a mineral substrate, hydroxyapatite (HA) particles, in the presence of the osteoinductive agent dexamethasone. When cells grown under these osteogenic conditions were analyzed for their osteogenic capacity, all six bone marrow specimens produced cells capable of osteogenic differentiation *in vitro* (as determined by ALP expression) and bone formation *in vivo* (subcutaneous implants of HA/cell constructs in athymic mice). However, after implantation of these HA/cell constructs into various human jawbone defects of the six patients, biopsy specimens taken at 4 months showed that bone formation by implanted cells was able to regenerate bone in only one of the six patients treated. The authors made the important observation that *in vitro* osteogenic assays and bone formation in preclinical mouse models may not necessarily correlate to successful bone regeneration in the more challenging clinical applications. They concluded further that inadequate vascularity could have resulted in the reported disappointing outcome of the study.[39] In accordance with the authors' conclusion from this study, it is our belief that not only is vascularization from the host environment essential to clinical bone regeneration, but even further, that the angiogenic potential of the transplanted cells themselves should play an active role in this vascularization process.

The BRCs used to treat the human jawbone defect in our study were not produced in the presence of any osteogenic factors, and a gelatin sponge (with no known osteoinductive or osteoconductive properties) was used as a carrier matrix to transplant the cells, as opposed to a mineralized matrix such as HA. Additionally, the biopsy specimen harvested at 6 weeks showed significant new bone formation containing abundant blood vessels. Although no direct evidence (i.e., labeling) is provided relative to the source of cells that produced the regenerated tissue, we make the assumption that the transplanted cells at least partly contributed to the regeneration because the bone core specimen analyzed was harvested from the central region of the defect and graft site.

We were able to identify this exact region through the use of surgical measurement templates/guides. Yet, even still, despite these promising clinical results, the fact that they were obtained in a single patient can be viewed as a study limitation and minimizes the general conclusions that can be drawn. An additional note is that this case presentation is part of a larger, U.S. FDA-regulated, randomized, controlled Phase I/II trial where a larger number of patients are to be treated with BRCs. This larger study is still ongoing as it includes a 1-year patient follow-up; however, upon study completion, all the clinical data will be analyzed and the results outlined in a future report. While it is realized that the feasibility of this process for routine tooth extraction surgeries is most likely not practical, this study was conducted as an FDA Phase I/II study to examine safety and efficacy of this therapy for regeneration of craniofacial bone. If results are favorable, this type of therapy may certainly be feasible for larger, more challenging craniofacial reconstructions.

Conclusion

There is a growing interest in cell therapy strategies to regenerate craniofacial tissues, particularly bone. However, key questions to be considered in utilizing these strategies are the following: What is the source of cells used in these approaches? How will the cells be processed and expanded to reach appropriate cell numbers for clinical applications? What is the phenotype and regenerative capacity of the cells produced? Through the current investigation, we report a novel approach to craniofacial regeneration with the utilization of an automated, closed-system, cell expansion process for the clinical-scale production of autologous cells, enriched in mesenchymal and vascular cell phenotypes. Additionally, it was demonstrated clinically that these cells possess the capacity to give rise to highly vascular bone, 6 weeks after transplantation into a jawbone defect. Although the findings presented herein cannot fully elucidate the answers to the aforementioned questions, they provide important insight toward that end.

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Address correspondence to:
Darnell Kaigler, D.D.S., M.S., Ph.D.
Department of Periodontics and Oral Medicine
University of Michigan
1011 N. University
Ann Arbor, MI 48109

E-mail: dkaigler@umich.edu

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