ORIGINAL ARTICLE

Evaluation of Autologously Derived Biomaterials and Stem Cells for Bone Tissue Engineering

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Despite progress, clinical translation of tissue engineering (TE) products/technologies is limited. A significant effort is underway to develop biomaterials and cells through a minimally modified process for clinical translation of TE products. Recently, bone marrow aspirate (BMA) was identified as an autologous source of cells for TE applications and is currently being tested in clinical therapies, but the isolation methods need improvement to avoid potential for contamination and increase progenitor cell yield. To address these issues, we reproducibly processed human peripheral blood (PB) and BMA to develop autologously derived biomaterials and cells. We demonstrated PB-derived biomaterial/gel cross-linking and fibrin gel formation with varied gelation times as well as biocompatibility through support of human bone marrow-derived stem cell survival and growth *in vitro*. Next, we established a plastic culture-free process that concentrates and increases the yield of CD146⁺/CD271⁺ early mesenchymal progenitor cells in BMA (concentrated BMA [cBMA]). cBMA exhibited increased colony formation and multipotency (including chondrogenic differentiation) *in vitro* compared with standard BMA. PB-derived gels encapsulated with cBMA also demonstrated increased cell proliferation and enhanced mineralization when assessed for bone TE *in vitro*. This strategy can potentially be developed for use in any tissue regeneration application; however, bone regeneration was used as a test bed for this study.

Keywords: peripheral blood, BMA, fibrin, concentrated BMA, CD146⁺/CD271⁺ cells, minimally modified tissue, clinical translation, intra-operative strategy, bed side TE

Impact Statement

Tissue engineering (TE) is a rapidly growing field; however, clinical translation of TE products remains challenging. Autologously sourced bone marrow aspirate (BMA) and peripheral blood (PB) offer relative ease of harvesting and host compatibility and require minimal/no regulatory approvals. In this study, concentration of BMA led to the enrichment of early progenitor cells, which can potentially improve TE outcomes. Furthermore, concentrating BMA in combination with calcium-mediated, cross-linked fibrin gels derived from PB presented enhanced proliferation and osteogenic potential for a minimally modified bone TE strategy. These findings demonstrate the potential utilization of BMA in combination with PB for development of clinically translatable TE strategies for regeneration of different tissues.

Introduction

TISSUE ENGINEERING (TE) is an alternative strategy to treat the loss or damage of tissues and organs.^{1,2} Numerous TE strategies have been developed using engineered biomaterials in combination with culture-expanded cells.³⁻⁷ Current efforts utilize patient-derived and minimally modified tissue sources to develop autologous TE strategies that can be more easily translated to the clinic.⁸⁻¹⁰

Biodegradable natural and synthetic biomaterials can be engineered into three-dimensional porous structures for tissue repair and regeneration.¹¹⁻¹³ Hydrogel biomaterials are especially desirable for TE due to their tissue-like characteristics, which include tissue-like mechanical properties, cell encapsulation ability, and high water content.¹⁴⁻¹⁶ Fibrin (FB), a nonglobular protein responsible for blood clotting, has been heavily researched in the last decade as an alternative to collagen for creating biodegradable hydrogels because of its major

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role in cellular interactions and wound healing.^{17–20} Despite the interest in fibrin as an alternative biodegradable material, the use of allogenic thrombin required for fibrinogen cross-linking has potential for negative immune responses and risk of disease transmission.^{21,22} Fibrin-based biomaterials/matrices can support cell growth and tissue regeneration; however, clinical translation remains a challenge as the currently available fibrin-based materials are derived from allogenic sources.^{23,24} Alternatively, fibrin hydrogels can be readily developed from autologous sources such as peripheral blood (PB) and bone marrow aspirate (BMA) and evaluated for TE *in vitro* and *in vivo*.^{25,26} Fibrin gels encapsulating chondrocytes, bone marrow-derived mesenchymal stem cells (BMSCs), fibroblasts, and osteoblasts have demonstrated that these biomaterials promote cellular adhesion and support proliferation, growth, and respective tissue differentiation.^{27–33} Our own work utilized fibrin in combination with hyaluronic acid as a hybrid gel matrix for cartilage and bone TE.^{34,35}

In TE, many cell sources are considered to provide progenitor or mesenchymal stem cells required for regeneration. Upon isolation, these cells are subjected to extended *ex vivo* culture to obtain the required number of cells or to prime cells for a desired application. The potential of these cells for tissue regeneration has been demonstrated *in vitro* and *in vivo*; however, these TE products need to go through extensive regulatory processes as the cells are processed/modified through *ex vivo* culture. The current focus is developing alternative culture methods to derive cells needed for TE applications, and BMA has been established as a viable cell source.³⁶ Autologous BMA is currently used by orthopedic surgeons to treat fractured bones through direct infusion of BMA onto allografts or autografts.³⁷ The advantage of using BMA is that it is readily available and can be harvested from either the iliac crest or any of the long bones.^{37,38} The bone marrow niche is also a rich source for several progenitor cells, such as BMSCs, hematopoietic stem cells, and endothelial progenitor cells, as well as growth factors, such as bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF). However, the volumes of BMA required to fill large or segmental defects do not contain adequate numbers of progenitor cells. This progenitor cell deficiency causes limited tissue formation.⁶ To overcome this challenge, researchers and clinicians have examined the possibility of concentrating BMA (cBMA) for progenitor cells through centrifugal and selective magnetic isolation methods.^{39–42} Studies have shown the potential of cBMA for bone, tendon, and ligament tissue regeneration.^{37,43–45}

In this study, we developed and characterized biomaterials and progenitor cells from human PB and BMA, demonstrating a clinically translatable, completely intraoperative tissue engineering strategy (CITES) for bone TE.

Materials and Methods

Bone marrow aspirate processing and peripheral blood plasma isolation

Freshly isolated human BMA was purchased from Lonza (20- to 30-year-old donors, males and females) and delivered to our laboratory within 24 h. Autologous human PB was purchased from Zen-Bio, Inc., (40- to 50-year-old donors, males and females) and mixed with an anticoagulant. BMA

and PB were both processed using the Magellan[®] system (Arteriocyte, Hopkinton, MA) according to the company's procedures.⁴⁶ Briefly, for BMA processing, a minimum of 30 mL of freshly isolated BMA was drawn into a 60-mL syringe primed with an anticoagulant. The BMA-containing syringe was then attached to the proximal end of the filter apparatus and BMA was gently pushed into the filter. When the marrow reached the end of the distal filter tubing, a gentle pull back on the plunger of the 60-mL syringe was provided to ensure that the entire volume was drawn. The 60-mL syringe was then attached to the syringe pump on the Magellan machine. The procedure began with the pump being activated to empty the syringe. Once the entire volume was loaded, the sample was subjected to a two-step centrifugation process. Step 1 was at 2800 rpm to separate and remove the red blood cell fraction and, in step 2, the remaining BMA was further separated into platelet-poor plasma (PPP) and platelet-rich plasma (PRP) fractions at 3800 rpm. This was a continuous process in which all three different fractions of BMA were collected into individual tubes. In our study, PRP isolated from BMA is referred to as cBMA. Unprocessed BMA was used as control. PB was processed similarly, in which the PPP layer was isolated and used for subsequent PB plasma gelation.

Blood plasma gelation

A range of CaCl₂ concentrations (5, 15, 25, 35, 45, 55, 65, 75, 85, 100, 200, 300, 400, and 500 mM) was prepared, 50 μ L of which was added to 100 μ L of PB plasma to observe gelation times at room temperature using 2-mL Eppendorf tubes. The degree of PB-fibrin gelation was evaluated by completely inverting the tubes containing plasma and observing gel formation, as shown in Figure 2. Three regions were identified: no gelation (if plasma remained in a fully liquid state), partial gelation (if plasma became more viscous, but did not fully adhere to the top surface when inverted), and substantial amount of gelation (if complete gelation was observed with the gel fully adhered to the surface). Commercially available FB hydrogels were used as controls and prepared by mixing 20 U/mL thrombin (Sigma-Aldrich) with 50 μ L of fibrinogen (Sigma-Aldrich). For cell studies, BMA or cBMA was added before addition of thrombin or CaCl₂.

Human bone marrow-derived mesenchymal stem cells from concentrated bone marrow aspirate culture

Human BMSCs (hBMSCs) were obtained from cBMA by seeding onto 150-mm culture dishes and cultured at 37°C in a humidified atmosphere containing 5% CO₂. Growth media consisted of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12; Life Technologies) with Glutamax, 10% fetal bovine serum (FBS; Life Technologies), and 100 U/mL penicillin–streptomycin (P/S; Life Technologies). Nonadherent cells were removed after 4 days with gentle washes of phosphate-buffered saline (PBS). Culture media were changed every 3 days and cells were passaged when plates were at 90% confluence. Cells were either used for experiments or cryopreserved for subsequent experiments.

Flow cytometry

For fresh (uncultured) BMA and cBMA, suspensions were first lysed (lysing buffer; BD) to eliminate red blood

cells. A MACSQuant flow cytometer (Miltenyi Biotec) was used. Suspensions were then incubated with the following markers: PE-Cy7-conjugated Hu CD 271, brilliant violet 421-conjugated Hu CD146, PE-conjugated Hu CD34, and FITC-conjugated Hu CD45. Unstained cells, live/dead, isotype controls, compensation beads, and fluorescence minus one (FMO) were used as controls and for gating purposes. PE was excited by the 488 nm laser and detected using a 575/25 band pass filter. The Live/Dead[®] Fixable Far Red Dead Cell Stain was excited by the 633 nm laser and detected using a 660/20 band pass filter.⁴⁷

Colony-forming unit assay

Colony-forming unit-fibroblast (CFU-f) assay was carried out by plating 25 μ L of cBMA and BMA separately into 24-well culture plates. After incubation for 7 days, cells were washed with PBS and then stained with 0.5% crystal violet for 10 min, rinsed several times with distilled and deionized water, and photographed to visualize CFUs of hBMSCs, as previously reported.^{48,49} The colony area was quantified using ImageJ (Fiji, National Institutes of Health, Bethesda, MD).

Trilineage differentiation

Uncultured cells (25 μ L/sample) were maintained in osteogenic media for 21 days using 24-well tissue culture plates. The media consisted of DMEM/F12 (Life Technologies) with Glutamax supplemented with 10% FBS, 1% P/S, 10 mM β -glycerol phosphate, 50 μ g/mL ascorbate-2-phosphate, and 10 nM dexamethasone. At day 21, Alizarin red staining was used to identify mineral deposition or calcium deposition. This colorimetric assay is based on the solubility of the Alizarin red matrix precipitate with cetylpyridinium chloride (CPC; Sigma-Aldrich). Briefly, after culturing in osteogenic media for 21 days, samples were washed with distilled water and fixed with 70% ethanol at 4°C for 1 h. After the ethanol was removed and allowed to air-dry, samples were incubated with the Alizarin red dye (Sigma-Aldrich) for 10 min at room temperature. Samples were photographed for representative images of mineralization. The samples were washed multiple times to remove excess Alizarin red dye and incubated with 10% CPC for 30 min at room temperature to solubilize the dye-matrix complex. The absorbance of the resulting solution was then read using a spectrophotometric plate reader at wavelength of 562 nm.^{50,51}

Uncultured cells (200 μ L/sample) were maintained in chondrogenic media for 14 days in 2-mL Eppendorf tubes. High-density pellet cultures were treated with chondrogenic conditioned media consisting of high-glucose DMEM/F12 with Glutamax, supplemented with 10 nM TGF- β_1 , ITS⁺, 50 μ g/mL ascorbate-2-phosphate, 100 μ g/mL Na-pyruvate, 40 μ g/mL proline, 100 nM dexamethasone, and 1% P/S, and cultured for 14 days.^{42,52} The resulting pellets were fixed, paraffin embedded, sectioned, and stained with 1% Alcian blue solution to examine qualitatively sulfated glycosaminoglycan production as a marker for chondrogenic differentiation.⁵²

Uncultured cells (25 μ L/sample) were maintained in adipogenic media using 24-well tissue culture plates. Cells were cultured for a 72-h cycle in DMEM/F12 with Glutamax media containing 10% FBS, 1% P/S, 0.2 mM indomethacin, 50 μ g/mL ascorbate-2-phosphate, 1 nM dexamethasone, 0.1 mg/mL

insulin, and 1 mM isobutyl-1-methylxanthine (IBMX). This was followed by culturing in maintenance media containing 10% FBS, 1% P/S, and 0.1 mM insulin for a 24-h cycle. These cycles were repeated four times, and then cells were fixed and stained with 0.2% Oil red O solution to visualize the formation of fat vacuoles.

Live-dead assay and cell proliferation

Live-dead cell viability assay (Invitrogen) was performed according to the manufacturer's protocol to label live cells with calcein AM and dead cells with ethidium homodimer-1 used to monitor cell growth and survival. Imaging was carried out using a confocal microscope on days 1, 7, 14, and 21 for cultured cells and on day 21 for uncultured cBMA using 24-well tissue culture plates.⁵³ To examine cell proliferation, samples were evaluated quantitatively using the Quant-iT[™] PicoGreen double-stranded DNA (dsDNA) assay (Invitrogen).^{50,52} The fluorescence of samples and standards was measured at 485/535 nm using a BioTek plate reader.

Extracellular calcium deposition

cBMA (25 μ L/sample), BMA (25 μ L/sample), and cultured hBMSCs (30,000 cells/sample) isolated from cBMA were cultured in osteogenic media for 21 days and encapsulated in PB-fibrin and FB gels using 24-well tissue culture plates. Tissue culture polystyrene (TCPS) was used as a control. Matrix mineralization or calcium deposition was then evaluated using Alizarin red staining, as described in the Trilineage Differentiation section.

Statistical analysis

For cell proliferation studies, data are reported as mean $\pm$ standard deviation. Comparisons between groups were performed using a simple Student's *t*-test or two-way analysis of variance, followed by Tukey's multiple comparison test, with statistical significance evaluated at $p < 0.05$. Three samples per group ($n = 3$) were analyzed at each time point for cell proliferation, CFU assays, trilineage differentiation assays, and calcium deposition, as well as for PB plasma gelation studies.

Results

The goal of our study was to determine the potential of autologously derived and processed PB as a biomaterial and BMA as a progenitor cell source for TE. We processed and concentrated PB and BMA using an automated centrifugation device to prepare samples for testing, as shown in Figure 1.

Processed PB was subjected to calcium-mediated cross-linking to develop an autologous biomaterial. To establish the optimal concentration of Ca²⁺ required for plasma gelation, a range of CaCl₂ concentrations was mixed with PB plasma and monitored for gelation (Fig. 2). Based on the gel formation behavior, three regions were identified: gelation (G), partial gelation (P), and no gelation (N) (Fig. 2A). CaCl₂ concentration ranges, 5–15 and 200–500 mM, conferred mostly no gelation (Fig. 2B). However, 25–100 mM CaCl₂ showed partial to substantial gelation. Overall, the trend we found was that 25–65 mM CaCl₂ exhibited a

FIG. 1. Schematic showing intraoperative processing of PB and BMA to develop patient-derived biomaterials and stem cells, respectively, for bedside tissue engineering. BMA, bone marrow aspirate; PB, peripheral blood; PPP, platelet-poor plasma; PRP, platelet-rich plasma. Color images are available online.

substantial amount of gelation, with 45 mM exhibiting gelation occurring in the shortest amount of time. Therefore, all subsequent analyses were done using this CaCl_2 concentration for PB-derived fibrin gel (PB-fibrin) development.

Next, the PB-fibrin gel's ability to support cell survival and growth was evaluated. hBMSCs isolated from cBMA were cultured *in vitro* to expand, subsequently encapsulated within PB-fibrin gels, and assayed for cell survival and proliferation (Fig. 3). Confocal images of live/dead staining, as a measure of cell survival, showed that hBMSCs seeded and cultured on PB-fibrin supported cell attachment from day 1 through 14 and exhibited continued cell growth and proliferation up to day 21 (Fig. 3A). These data were confirmed by cellular proliferation studies that compared the DNA content of cells cultured in PB-fibrin gels with commercially obtained FB gels and TCPS. FB gels were used as a positive control, while TCPS served as the experimental control. Results from the proliferation assay demonstrate that like the controls, PB-fibrin gels supported cell growth throughout the 21-day culture period with significantly increased DNA content compared with FB gels at days 1, 7, and 14 (Fig. 3B).

BMA and cBMA were evaluated for enrichment of progenitor cells. The cell yield from fresh BMA and cBMA was

7.78 million cells/mL and 6.82 million cells/mL, respectively. Although the cell numbers do not vary greatly before and after concentration, it is important to note that concentration of BMA leads to differences in cell composition and the number of cells that can potentially form MSC colonies/become MSCs. Therefore, to compare their performance in terms of colony-forming ability and trilineage differentiation, a fixed volume of fresh and concentrated BMA was used for this purpose. Next, flow cytometry of hBMSCs from fresh BMA and cBMA was used to measure expression of early progenitor surface markers, CD146 and CD271 (Fig. 4). BMA cells were 87.5% $\text{CD146}^-/\text{CD271}^-$ double negative, whereas cBMA cells were only 62.1% double negative. cBMA also contained 1.09% CD271^+ and 35.5% CD145^+ cells, whereas BMA cells were only 0.92% and 11.5% single positive for CD271^+ and CD145^+ , respectively. Encouragingly, $\text{CD146}^+/\text{CD271}^+$ double-positive cells were almost 10-fold higher in cBMA compared with BMA, 1.26% versus 0.13%, respectively.

To observe cell growth of the unprocessed/fresh BMA and cBMA, we measured colony-forming ability after 7 days of culture in a CFU-f assay (Fig. 5). cBMA cells exhibited a larger number of colonies with higher intensity staining than the BMA group, which displayed only a few

FIG. 2. Optimal PB plasma gelation occurs within a specific range of CaCl_2 concentrations. **(A)** Representative images of no gelation (*left*), partial gelation (*middle*), and gelation (*right*) of PB plasma facilitated by CaCl_2 . **(B)** Analysis of optimal CaCl_2 required to facilitate gelation. CaCl_2 ranging from 5 to 500 mM was added to PB plasma to examine gelation time. $n=3$ replicates/group. Color images are available online.

FIG. 3. PB-fibrin gels support precultured cBMA cell proliferation and survival over 21 days of culture and exhibit higher proliferation compared with FB gels. Proliferation and survival of cultured hBMSCs isolated from cBMA using imaging and DNA content measurements. **(A)** Live/dead assay of cells cultured in PB-fibrin at days 1, 7, 14, and 21; *green* represents live cells and *red* represents dead cells. Images were recorded at 10 $\times$ magnification. **(B)** Proliferation of hBMSCs cultured in PB-fibrin, fibrin, and TCPS plates at days 1, 3, 7, 14, and 21 assessed by PicoGreen DNA content ($\mu\text{g}/\mu\text{L}$) assay. $n=3$ replicates/group/time point. $*p<0.05$ for PB-fibrin or TCPS versus fibrin alone assessed by two-way ANOVA with Tukey's multiple comparison test. ANOVA, analysis of variance; cBMA, concentrated BMA; FB, fibrin; hBMSCs, human bone marrow-derived mesenchymal stem cells; TCPS, tissue culture polystyrene. Color images are available online.

FIG. 4. cBMA contains more early progenitor hBMSCs than BMA alone. Flow cytometry analysis of fresh uncultured hBMSCs from BMA and cBMA (concentrated using MAGELLAN[®]) using early MSC markers, CD271 and CD146. The *x*-axis represents CD146⁺ cells, *y*-axis represents CD271⁺ cells, and *upper-right quadrant* represents cells double positive for CD271 and CD146. Color images are available online.

FIG. 5. cBMA hBMSCs produce more colonies than BMA hBMSCs. CFU-f assay of hBMSCs from BMA and cBMA cultured for 7 days and stained with *crystal violet*. (A) Scanned images of colony formation (*blue*), (B) number of colonies, and (C) area (μm^2) per colony in each group. Quantification performed using ImageJ software. $n=3$, $**p<0.01$ BMA versus cBMA by Student's *t*-test. CFU-f, colony-forming unit-fibroblast. Color images are available online.

colonies with weak staining (Fig. 5A). Quantification of colony number and area using ImageJ confirmed significantly more colonies and activity (area) for cBMA cells compared with BMA cells (Fig. 5B, C).

We next evaluated the multilineage potential or multipotency of cBMA and BMA using trilineage differentiation assays, where hBMSCs isolated from each group were cultured in osteogenic, chondrogenic, and adipogenic conditions (Fig. 6). BMA cells exhibited sparse staining under osteogenic and adipogenic conditions and could not be grown at all in chondrogenic conditions; chondrogenic samples from BMA samples did not form a pellet and therefore no chondrogenic differentiation was observed. In contrast, cBMA cells had clear high-intensity staining with more areas of Alizarin red, Oil red O, and Alcian blue staining compared with BMA cells, indicating enhanced osteogenic, adipogenic, and chondrogenic differentiation, respectively. Clear and uniform cBMA hBMSC differentiation in all three lineages demonstrates more robust multipotency than BMA hBMSCs.

With the establishment of cBMA multipotency, we tested and compared cBMA cell survival and growth when encapsulating in PB-fibrin or commercial FB, or when cultured on TCPS for 21 days (Fig. 7). Live/dead staining of cBMA showed that all groups supported high density and confluency of cells with uniform distribution at day 21, with PB-fibrin exhibiting a more organized cell layer within the gel (Fig. 7A). These results were supported by the proliferation studies, with similar DNA content among all groups on day 1 and decreased DNA content up to day 7 (Fig. 7B). However, at later time points, days 14 and 21, the PB-fibrin

group showed a trend toward higher cell proliferation compared with FB gels and TCPS.

Finally, we measured the osteogenic potential of cBMA and BMA using PB-fibrin. Gels were loaded with cultured hBMSCs, BMA, or cBMA, cultured in osteogenic media for 21 days, and evaluated for subsequent extracellular calcium deposition (Fig. 8). FB gels, TCPS, and cultured hBMSC groups were used as controls. In the comparative differentiation study within the cBMA culture, PB-fibrin gels exhibited significantly higher calcium deposition at day 21. In addition, cBMA was superior to BMA in both PB-fibrin and FB groups. Cultured hBMSCs showed better osteogenic potential than cBMA; however, within this group, PB-fibrin showed higher calcium deposition compared with FB and TCPS.

Discussion

Blood or BMA-derived biomaterials, cells, and growth factors have been widely studied as natural autologous sources for tissue repair and regeneration.^{43,54–60} These materials offer beneficial properties for TE, advantages of which include ease of availability (obtained from human plasma), biocompatibility, decreased risk of immunogenicity, and minimal manipulation required for use, thus reducing FDA hurdles and facilitating clinical translation. In this study, both PB and BMA were processed using a single closed-loop unit, through a minimally manipulated process with a fully automated device, MAGELLAN. This device is a closed system with the ability to support rapid concentration and separation of the different layers of PB and BMA with reproducible

FIG. 6. cBMA hBMSCs exhibit increased multipotency compared with BMA hBMSCs. Trilineage assessment—osteogenic, adipogenic, and chondrogenic differentiation—of cultured hBMSCs from BMA and cBMA. Scanned images of osteogenic and adipogenic differentiation cultures of BMA and cBMA observed by Alizarin red and Oil red O staining, respectively. BMA hBMSCs did not differentiate in chondrogenic conditions (labeled as not available; N/A), but a histological image of cBMA hBMSCs in chondrogenic conditions shows sulfated GAG patterns by Alcian blue staining (scale bar = 200 μ m). GAGs, glycosaminoglycans. Color images are available online.

FIG. 7. PB-fibrin gels support nonprecultured cBMA cell proliferation and survival over 21 days of culture. Nonprecultured cBMA cells were encapsulated in PB-fibrin, fibrin, or directly on TCPS, cultured for 21 days, and assayed. (A) Live/dead assay of cells cultured at day 21; green represents live cells and red represents dead cells. Images were recorded at $10\times$ magnification. (B) Proliferation of hBMSCs cultured in PB-fibrin, fibrin, and TCPS plates at days 1, 3, 7, 14, and 21 assessed by PicoGreen DNA content ($\mu\text{g}/\mu\text{L}$) assay. $n=3$; * $p<0.05$ by two-way ANOVA with Tukey's multiple comparison test. Color images are available online.

results. Plasma obtained from PB was used to develop fibrin biomaterial/gel, while BMA was processed using the same device to produce concentrated BMA—cBMA—to improve MSC and progenitor cell yields. Consistent with the literature, PPP from PB and PRP from BMA were chosen to derive fibrin biomaterial/gel and stem cells, respectively.^{25,61}

Blood/bone marrow-derived plasma conversion to fibrin and its gel physical/biological properties are often controlled by varying the amount of thrombin added.^{25,56} In our study, we chose CaCl_2 to control these properties and established an optimal concentration required for PB plasma gelation, which is important for determining gelation quality and

FIG. 8. Fresh cBMA within PB-fibrin has higher osteogenic potential than fibrin or TCPS alone. Osteogenic potential was determined measuring extracellular calcium deposition after 21 days of culturing *in vitro*. (A) Scanned images of Alizarin red staining in each culture. (B) Quantification of Alizarin red staining of cultured hBMSCs isolated from cBMA, BMA, or cBMA cultured in PB-fibrin, fibrin, or TCPS. $n=3$, * $p<0.05$ by two-way ANOVA with Tukey's multiple comparison test. Color images are available online.

timing. We found that 25–100 mM CaCl_2 demonstrated partial to substantial gelation, and 45 mM CaCl_2 exhibited a substantial amount of PB plasma gelation within 3.5 h. For the PB-derived plasma gel to be used intraoperatively, there may be a need to consider addition of thrombin to reduce the time of gelation. Further studies are required to evaluate the optimal amount of thrombin addition to allow for suitable gelation times for intraoperative use.

Blood coagulation is a complex process involving an activation cascade of various coagulation factors.⁶² Blood-derived plasma developed into fibrin gels through enzyme-activated (thrombin) or chemical-activated (CaCl_2) methods exhibits cell compatibility and growth *in vitro* and *in vivo*.^{29,56,63} In our study, we used a calcium-activated approach to convert PB plasma into fibrin gel. Ca^{+2} ions can mediate activation of the prothrombinase complex (a combination of coagulation factors XIII, Xa, and Va), which activates autologously present thrombin to convert fibrinogen to fibrin.^{62,64} This approach avoids the use of allogenic thrombin, so a biomaterial with no immunogenic concerns could be used to develop clinically translatable TE products.

PB-derived fibrin biomaterial/gel was assessed for basic biomaterial compatibility by encapsulating cultured hBMSCs and measuring cell proliferation. Higher cell proliferation of PB-fibrin cells compared with control fibrin at all time points is likely due to differences in the composition of the two fibrin-based matrices. PB-derived fractions, namely PRP and PPP, are known to contain GFs that aid in cell proliferation and differentiation, such as TGF- β , IGF-1, and PDGF.^{65–68} In this study, we consolidated PPP to develop PB-derived fibrin gels through the addition of CaCl_2 . By doing so, it is likely that some of these GFs are retained as part of the matrix helping to achieve the higher rates of proliferation, which are otherwise absent from the commercially available fibrinogen-derived fibrin. This is further supported by the cell yield of PPP, roughly 350,000 cells/mL (data not shown), which is likely the source of the GFs as part of the matrix. These results are in line with other studies in which autologous fibrin clots derived from both PB and BMA supported cell proliferation and osteogenic differentiation with differences in efficiency based on the amount of growth factors present.^{69,70}

BMA is increasingly utilized in TE and regenerative medicine; however, the major limitation is the rarity (or low cell number) of nonhematopoietic MSCs, constituting only 0.001–0.01% of BMA.⁷¹ To overcome this, BMA can be concentrated using different strategies and automated devices, effectively increasing the number of BMSCs. Unlike plastic-adhered and cultured MSCs, concentrated BMA presents early MSC progenitors, which display completely different surface markers.^{72–76} In this study, cells isolated from BMA and cBMA were characterized using CD271 and CD146 surface markers, which are established early progenitor cell markers. Flow cytometry revealed that concentration of BMA led to substantial enrichment of cells, with an almost 10-fold increase in double-positive CD146⁺/CD271⁺ progenitor cells, compared with nonconcentrated BMA. Further evaluation demonstrated superior colony-forming ability (CFU-f), confirming the enrichment of this desirable progenitor cell population. The CFU-f enrichment assay is widely used as a functional assay to quantify

progenitor cells as the characteristic features of MSCs are adherence and generation of colonies when plated at low densities on TCPS.⁷⁷ CFU-f demonstrated a greater number of progenitor cell colonies and colony area for cBMA compared with BMA, confirming that cBMA provides a richer source of progenitor cells.

To compare the multipotency of BMSC populations, cBMA and BMA were subjected to trilineage differentiation, which involves three separate cultures to induce mesenchymal stem cells into three major tissues in the body: bone, cartilage, and fat.^{12,78} In this assay, we observed increased adipogenic and osteogenic differentiation as well as the presence of chondrogenic differentiation in cBMA versus BMA, which based on our hBMSC enrichment data, was not surprising. Chondrogenesis was not possible with BMA due to the low progenitor cell number. Overall, our studies indicated that concentration of BMA would lead to an increased number of colonies and potential for trilineage—including chondrogenic—differentiation. Therefore, it is plausible to speculate that this is due to the insufficient number of cells in unprocessed BMA and the higher numbers of progenitor cells in cBMA. Together, our results establish the cBMA as a more powerful cell source for TE applications.

After determining the enhanced functionality of each component, we combined the PB-derived biomaterial/gel and cBMA to assess cell proliferation. cBMA encapsulated within PB-fibrin gel or commercially available FB or cultured on TCPS showed decreased cell proliferation from days 1 to 7, which was recovered at the later time points, days 14 and 21. This is likely due to the heterogeneous mixture of hematopoietic cells within the PRP component of BMA, including erythrocytes, granulocytes, monocytes, lymphocytes, platelets, and peripheral stem cells.⁷⁹ At early time points, the majority of the cBMA consists of nonanchorage-dependent cells, namely platelets, which have a circulating life span of 7–10 days.⁸⁰ This decrease in cell proliferation at early time points corresponds to the loss of these cells, whereas the recovery of cell proliferation/DNA content corresponds to the increase in proliferation of hBMSCs.

To establish our method in the context of bone TE, we assessed the osteogenic potential of cBMA encapsulated within the PB-fibrin gel matrices. PB-fibrin gels consistently expressed higher levels of calcium deposition, compared with control FB gels, indicative of increased osteogenic potential. Differences in subsequent calcium deposition following a 21-day culture are likely due to differences in culture conditions, namely additional growth factors. The PB-fibrin gel exhibited increased Alizarin red staining, suggesting that growth factors retained within this matrix are responsible for promoting enhanced osteogenic differentiation and mineralization. Further studies will need to be performed to confirm these findings.

TE strategies for restoration of damaged tissue require the use of scaffold matrices in combination with relevant cell sources. These biomaterials and cells are often of synthetic origin or are modified extensively, thus requiring FDA approval for implantation. In this study, we present a minimally modified process in which PB and BMA collected from a patient can be processed intraoperatively using an automated device to acquire an autologously enriched cell

population and biomaterial matrix. Using these in unison, a CITES can be developed and implemented for tissue repair/regeneration.

Conclusions

Our study established the feasibility of developing biomaterials and cells from autologous tissue sources using a consistent and reproducible process. The autologously derived and cross-linked fibrin biomaterial/gel demonstrated biocompatibility by supporting hBMSC survival and growth *in vitro*. BMA concentration studies revealed a 10-fold increase in progenitor cells without the use of conventional plastic culture. Increased number of colonies and enhanced trilineage differentiation, in particular chondrogenic differentiation ability, clearly show increased regenerative potential of cBMA over standard BMA. For the first time, this study successfully demonstrated a bone TE strategy utilizing autologously developed biomaterials and cells. In conclusion, our study proposed and implemented a CITES that could accelerate clinical translation of a TE product.

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