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Cryopreserved bone marrow aspirate concentrate as a cell source for the colony-forming unit fibroblast assay

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ABSTRACT

Purpose: The prevalence of connective tissue progenitor cells within a cell-based therapy is often quantified using the colony-forming unit fibroblast (CFU-F) assay. The present study investigates the feasibility of using cryopreserved bone marrow aspirate concentrate (BMAC) as an alternative cell source to fresh BMAC for CFU-F quantification.

Methods: Freshly prepared and corresponding cryopreserved BMAC samples from patients receiving autologous cell therapy (n = 98) were analyzed using the CFU-F assay for comparison. Cultures were established by directly plating BMAC at low cell densities and maintained for a 2-week growth period. Colonies were enumerated to determine CFU-F frequency, and a subset of cultures was imaged and analyzed to quantify colony area and density.

Results: A nonlinear relationship was observed between plating density and CFU-F frequency over a wide range in plating densities (~30-fold). Cryopreserved BMAC yielded recoverable (77 ± 23%) and viable (73 ± 9%) nucleated cells upon thawing. After cryopreservation, CFU-F frequencies were found to be significantly lower (56.6 ± 34.8 vs. 50.3 ± 31.7 colonies per million nucleated cells). Yet the number of CFU-F in fresh and cryopreserved BMAC were strongly correlated (r = 0.87) and had similar area and densities. Further, moderate correlations were observed between the number of CFU-F and nucleated cells, and both the mean colony area and density were negatively correlated with patient age. Notably, no relationship was found between CFU-F frequency and age, regardless of whether fresh or cryopreserved BMAC was used.

Conclusions: Freshly prepared and cryopreserved BMAC yielded correlated results when analyzed using the CFU-F assay. Our findings support the cryogenic storage of patient BMAC samples for retrospective CFU-F analyses, offering a potential alternative for characterizing BMAC and furthering our understanding of progenitor cells in relation to clinical outcome.

Introduction

Orthopedic regenerative medicine is a rapidly growing medical specialty that uses both autologous and allogeneic orthobiologics to assist in tissue healing [1]. Autologous bone marrow stands as a common source of progenitor cells, and one predominant orthobiologic, bone marrow aspirate concentrate (BMAC), has been safely used to treat a variety of orthopedic conditions [2–4]. The existence of connective tissue progenitor cells within bone marrow was first described by Friedenstein and colleagues, when bone formation was observed within diffusion chambers populated with bone marrow cell suspensions and implanted intraperitoneally [5]. Later studies revealed these progenitor cells to adhere in culture when plated at low density and proliferate to form discrete colonies containing fibroblast-like cells [6,7], capable of differentiating into cells of different mesenchymal lineages, including bone, cartilage and fat, *in vitro* [8]. However, the *in vivo* multipotency of connective tissue progenitor cells has been disputed [9], and paracrine secretion of trophic and immunomodulatory factors is currently thought to be the primary mode of action [10]. Notwithstanding, several studies have now reported improved patient outcomes when autologous BMAC treatments with greater numbers of progenitor cells are used [11,12]. Unfortunately, point-of-care assays are not currently available to assess the number of progenitor cells within a BMAC therapy, hindering efforts to correlate their prevalence with patient outcomes.

A colony-forming unit fibroblast (CFU-F) assay adopts traditional cell culture techniques to determine the frequency of plastic adherent, connective tissue progenitor cells contained within a biological sample, such as BMAC. One important premise of the CFU-F assay is

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that each colony is generated from a single progenitor cell, and therefore, the number of colonies observed is representative of the number of progenitor cells as a fraction of the total cells plated [13]. Unfortunately, owing to the inherent nature of the CFU-F assay, results cannot be obtained *a priori* because patients are treated with autologous BMAC long before a clinician has knowledge of the progenitor cell composition. Moreover, fresh samples of BMAC must be assayed within a timely manner for every patient investigated, an untenable option for most clinicians because large studies would be encumbered with persistent periods of cell culture. As the field of orthopedic regenerative medicine advances, there is a growing need to improve upon the quantitative characterization of BMAC therapies. Cryopreserved BMAC, if similar to fresh BMAC with respect to culture performance, could serve as an alternative option to conduct the CFU-F assay once a retrospective cohort of patients is identified. This proposed methodology would minimize the overall assay time required (batch vs. continuous), minimize the number of samples to be analyzed (retrospective samples of interest vs. all treated patients) and additionally minimize sources of laboratory variation potentially inherent upon culturing samples at many time points.

Strong correlations have been observed between pre-freeze and post-thaw bone marrow across several cell populations, including mononuclear cells, committed myeloid precursors and hematopoietic stem cells [14]. Additionally, connective tissue progenitor cells have been recovered from cryopreserved bone marrow and successfully culture expanded with clinical applications in mind [15,16]. Although several groups have performed the CFU-F assay with both fresh and cryopreserved bone marrow and reported no differences between the two, data are limited [15] or not shown [17]. In the present study, patient-matched fresh and cryopreserved BMAC samples were compared using the CFU-F assay to determine whether the prospective cryopreservation of patient samples is a feasible storage strategy to employ when collecting samples to be used retrospectively for comparing the prevalence of connective tissue progenitor cells with patient outcomes.

Methods

BMAC

Patients undergoing orthopedic procedures with autologous BMAC for musculoskeletal injuries elected to have a small portion of their injectate retained for cellular analysis. Informed consent was obtained from all individual participants included in the study. Using sterile technique, bone marrow was harvested under ultrasound or fluoroscopic guidance and processed into BMAC, as previously described [4]. In brief, 5–10 mL of whole bone marrow aspirate (BMA) was harvested from each of 3–6 locations per side within the posterior superior iliac spine into syringes containing heparin. The BMA was serially centrifuged at 200g for 6 min, and the resultant nucleated cell–rich buffy coat was manually isolated using aseptic technique. Before clinical use, a small representative volume of BMAC ($\leq$ 0.2 mL) was collected for cell counting, cryopreservation and CFU-F cell culture. Patient age, gender, BMA volume and cell concentration, and BMAC volume and cell concentration are summarized in **Table I**.

Nucleated cell count

Complete blood counts were performed on all BMA and BMAC samples at the time of laboratory processing using an automated hematology analyzer (ABX Micros 60, Horiba Medical, Montpellier, France). Before counting, the BMAC samples were diluted tenfold in sterile saline to remain within the linear range of the instrument. The results from the white blood cell parameter were used to report total nucleated cell counts.

Table I
Patient and sample data presented as mean ± SD, median, and (minimum-maximum).

Patients	N = 98
Age (years)	48 ± 17, 53, (13–78)
Gender (male, female)	58 (59%), 40 (41%)
BMA (mL)	81 ± 20, 88, (22–120)
BMA (10^6 cells/mL)	25 ± 10, 23, (11–50)
BMAC (mL)	3.4 ± 1.7, 3.0, (0.9-8.7)
BMAC (10^6 cells/mL)	408 ± 113, 406, (113–672)

Cryopreservation of BMAC

Ten million nucleated cells from each BMAC sample (10–85 μL) were directly transferred into cryogenic vials and resuspended in chilled cryopreservation medium (1 mL) consisting of 63% minimal essential medium alpha (Corning, Corning, NY, USA), 1% L-alanyl-L-glutamine (Corning), 1% penicillin-streptomycin (GE Healthcare, Chicago, IL, USA), 30% fetal bovine serum (Access Biologicals, Vista, CA, USA) and 5% dimethyl sulfoxide (Corning). The cryogenic vials were immediately added to an isopropyl alcohol–based controlled rate freezing container and placed in a −80°C freezer. After 24 h, the BMAC samples were removed from the controlled rate freezing container and remained at −80°C for up to 1 week before being transferred to liquid nitrogen for cryogenic storage.

Cryorecovery of BMAC

Frozen BMAC samples were removed from cryogenic storage and gently swirled within a 37°C water bath for 2 min to permit rapid thawing. The contents of the thawed vials were subsequently diluted tenfold in warmed complete culture medium consisting of 88% minimal essential medium alpha, 1% L-alanyl-L-glutamine, 1% penicillin-streptomycin, 10% fetal bovine serum and 1 ng/mL human basic fibroblast growth factor (Gold Biotechnology, St. Louis, MO, USA). Nucleated cell count and viability measurements were obtained from each BMAC sample post-thaw using 0.4% Trypan Blue stain and an automated cell counter (TC20, BioRad, Hercules, CA, USA) before further dilution in complete culture medium for cell culture.

CFU-F assay

Initially, freshly prepared BMAC (n = 30) was serially diluted in complete culture medium and directly plated at densities of 90, 30, 10 and 3.3×10^3 nucleated cells per cm^2 within standard tissue culture six-well plates (Greiner Bio-One, Kremsmünster, Austria). In subsequent experiments, fresh and cryopreserved BMAC samples (n = 98) were plated at 30 and 10×10^3 nucleated cells per cm^2. Viability was taken into account when plating the thawed BMAC to ensure equivalent numbers of living nucleated cells in both the fresh and cryorecovered conditions. Following a 48 h attachment period, non-adherent cells were removed by washing with phosphate buffered saline (PBS), and the remaining cells were maintained at 37°C and 5% CO_2 with biweekly replacement of complete culture medium. After 2 weeks of culture, the six-well plates were washed with PBS and subsequently stained with 3% (w/v) crystal violet (Sigma Aldrich, St. Louis, MO, USA) in methanol (EMD Millipore, Burlington, MA, USA). Macroscopic colonies, greater than 1 mm in diameter and containing a minimum of 100 spindle-shaped cells (**supplementary Figure 1**), were identified and counted by three independent observers.

Image analysis of colony area and intensity

High-resolution images (1200 dpi) of a subset of six-well plates containing CFU-F from fresh and cryopreserved BMAC (n = 52) were

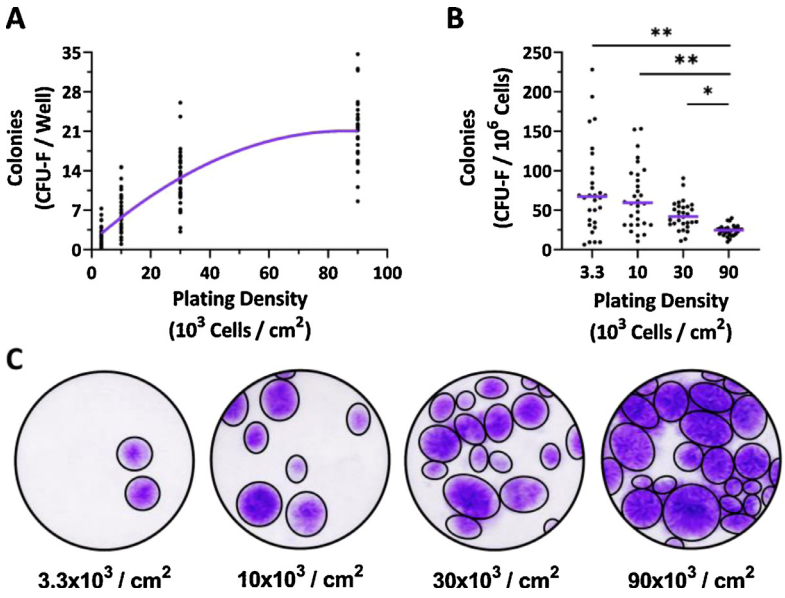

Figure 1. The relationship between plating density and colony formation in fresh BMAC samples is non-linear. (A) The average number of counted CFU-F per well plated within standard six-well plates at densities of 3.3, 10, 30 and 90 $\times$ 10^3 nucleated cells per cm^2; a quadratic least square regression illustrates the non-linear relationship; (n = 30 $\times$ 4). (B) Colony frequencies obtained by normalizing the average CFU-F per well to the input number of nucleated cells; lines represent the median; Friedman test with Dunn's multiple comparisons: $^{**}P < 0.001$, $^{*}P < 0.01$ versus 90 $\times$ 10^3 nucleated cells per cm^2. (C) Representative high-resolution well scans from a single donor; circles demarcate individual CFU-F as counted by one of three independent observers.

acquired using a commercial flatbed scanner (CanoScan LiDE220, Cannon, Tokyo, Japan). A java-based plugin (ColonyArea) for ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to automatically crop the images to individual wells, convert them to gray-scale and apply a background threshold for colony detection [18]. The mean colony area for each well was subsequently calculated by dividing the number of pixels having an intensity greater than zero by the total number of manually counted colonies and scaling the result to square millimeters. Similarly, the mean colony density for each well was calculated by dividing the raw integrated density (pixel area * mean gray value) of the thresholded regions by the number of manually counted colonies and scaling the result by a factor of 10^{-5}.

Statistical analysis

All colony data are presented as averages of the individual wells across a six-well plate, and all other data are presented as averages (mean $\pm$ SD) among patient BMAC samples. Normality was assessed using the D'Agostino-Pearson test, and the data were not normally distributed. Therefore, non-parametric statistical tests were performed (GraphPad Prism, GraphPad Software, La Jolla, CA, USA). Differences between multiple pairwise groups were evaluated using the Friedman test with Dunn's post hoc multiple comparison testing, differences between two pairwise groups were evaluated using the Wilcoxon signed rank test and strengths of association were evaluated using Spearman's rank-order correlation. Comparisons and associations were considered statistically significant at $P < 0.05$.

Results

Optimizing the plating density of BMAC for the CFU-F assay

A limited number of freshly prepared BMAC samples were plated over a 3-fold dilution series to determine the appropriate plating density, or densities, to evaluate CFU-F formation (**Figure 1**). When plated at 90, 30, 10 and 3.3 $\times$ 10^3 nucleated cells per cm^2 and cultured for a period of 2 weeks, a non-linear relationship between plating density and CFU-F count was observed (**Figure 1A**). After normalizing to the total number of input cells, the calculated CFU-F frequency (CFU-F per million nucleated cells) trended downward with plating density and was significantly lower at the highest density (**Figure 1B**). Representative high-resolution well scans show differences in colony formation across the investigated plating densities (**Figure 1C**). Overcrowding caused difficulty in discerning individual colonies at the highest plating density, whereas the majority of BMAC cultures (22 of 30) contained wells with one or no CFU-F at the lowest plating density. All BMAC samples consistently produced at least three CFU-F per well at one or both intermediate plating densities. Thus, 30 and 10 $\times$ 10^3 nucleated cells per cm^2 were selected as ideal plating densities to evaluate cryopreserved BMAC as a cell source for the CFU-F assay.

Cryopreserved BMAC yields viable nucleated cells

Small sample volumes of BMAC, placed within dimethyl sulfoxide–containing cryopreservation medium, subjected to a controlled rate freezing process, and cryopreserved for an average duration of 16 $\pm$ 14 days, produced viable nucleated cells upon thawing (**Figure 2**). An average of 77 $\pm$ 23% of cells, ranging from 34% to 160%, were recovered from a target number of 10 million cryopreserved nucleated cells (**Figure 2A**). Cell viability ranged from 49% to 88% with an average of 73% $\pm$ 9% (**Figure 2B**).

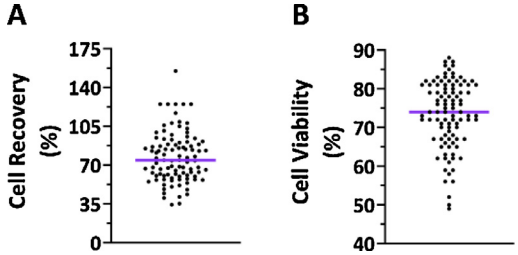

Figure 2. Viable nucleated cells are obtained from cryopreserved BMAC. (A) Cell recovery and (B) viability measurements following cryorecovery; lines represent the median (n = 98).

Cryopreserved BMAC as a cell source for the CFU-F assay

Fresh and corresponding cryopreserved BMAC samples were cultured at two plating densities to obtain CFU-F counts, and a subset of culture plates were scanned to quantify colony area and density (**Figure 3**). The lower plating density of 10×10^3 nucleated cells per cm^2 was used to calculate the CFU-F frequency of a BMAC sample only if there was an average of three or more CFU-F per well in both experimental conditions, otherwise the higher plating density was used (**supplementary Figure 2**). Approximately 40% (38 of 98) of BMAC samples required plating at 30×10^3 nucleated cells per cm^2 to calculate the CFU-F frequency using this criterion. Overall, compared with fresh BMAC, the CFU-F frequencies were lower within samples that underwent cryopreservation (**Figure 3A**). Average CFU-F frequencies of 50.3 ± 31.7 (0.0050% ± 0.0032%) and 56.6 ± 34.9 (0.0057% ± 0.0035%) were quantified in cryopreserved and fresh BMAC, respectively, representing a small, yet significant, pairwise difference (fold change 0.95 ± 0.38, $P < 0.001$).

A strong positive correlation ($r = 0.87$, $P < 0.001$) was observed between fresh and cryopreserved BMAC with respect to CFU-F frequency (**Figure 3B**). No significant differences in mean colony area or density were found following cryopreservation (**Figure 3C**). Moreover, fresh and cryopreserved BMAC samples were moderately correlated ($r = 0.49$, $P < 0.001$) with respect to colony area (**Figure 3D**) and density (data not shown). Representative images of CFU-F assay plates from a single donor illustrate the comparable nature of CFU-F quantified from fresh (**Figure 3E**) and cryopreserved (**Figure 3F**) BMAC samples.

Cryopreserved BMAC samples from a limited number of patients were thawed a second time, approximately 10 months (330 ± 30 days) after having been initially thawed, to investigate the effects longer periods of cryopreservation have on cell viability and CFU-F frequency. Viability was not significantly affected (75% ± 7% vs. 78% ± 7%), whereas CFU-F frequency remained strongly correlated ($r = 0.79$, $P = 0.005$) between the first and second instance of cryorecovery (**supplementary Figure 3**).

CFU-F, Nucleated cells and patient age

Nucleated cell and CFU-F counts within fresh BMAC samples were compared to determine their strength of association (**Figure 4**). The average concentrations of nucleated cells and CFU-F were $4.1 \pm 1.1 \times 10^8$ per mL and $2.3 \pm 1.6 \times 10^4$ per mL, respectively, and a moderate correlation ($r = 0.37$, $P < 0.001$) was observed between the two (**Figure 4A**). When accounting for the total volume of BMAC prepared, 3.4 ± 1.7 mL (**Figure 4B**), the strength of the positive correlation ($r = 0.64$, $P < 0.001$) between the overall number of CFU-F and total nucleated cells increased (**Figure 4C**). Similar trends were observed when using the CFU-F data acquired from cryopreserved BMAC samples (**supplementary Figure 4**).

Colony formation and CFU-F frequency were compared with patient age to determine whether any relationships exist (**Figure 5**). Patient age ranged from 13 to 78 years, with an average age of 48 ± 17 years. When the CFU-F frequency (**Figure 5A**) was compared with age, no correlation was present ($P > 0.05$). However, when normalized to the volume of bone marrow aspirated, a weak negative trend ($r = -0.19$, $P = 0.068$) emerged (**Figure 5B**). Moreover, moderate negative correlations ($r \approx -0.3$, $P < 0.05$) with age were observed for both mean colony area (**Figure 5C**) and density (**Figure 5D**). The CFU-F data obtained from cryopreserved BMAC samples yielded similar results (**supplementary Figure 5**).

Discussion

In the present study, both freshly prepared and cryopreserved BMAC from the same group of patients were evaluated by the CFU-F assay and compared to determine the feasibility of using cryopreserved samples to estimate the connective tissue progenitor cell content of bone marrow–based treatments. A direct plating method was used to quantify CFU-F because others have reported commonly used density gradient separation techniques reduce the recovery of progenitor cell populations [19–21]. Our results demonstrate a non-linear relationship between plating density and colony formation, challenging a seminal report describing the relationship as linear [7]. However, culture duration is a contributing factor, and our longer period (14 vs. 8 days) could allow adjacent colonies to fuse to a greater extent, resulting in the underestimation of CFU-F at higher plating densities. In support of this, a decrease in CFU-F counts between days 7 and 10 of culture with higher plating densities has been reported [22], and after normalizing to the input number of cells from directly plated bone marrow, the relationship between plating density and CFU-F frequency has been described as negatively correlated [20,22].

Every BMAC sample was plated at two densities, one three times greater than the other, to quantify colonies in donors having a wide range of CFU-F frequencies, as recommended in the literature [22]. The lower plating density was selected based on both the results from our initial dilution series and previous work identifying 10×10^3 cells per cm^2 as the most advantageous density for plating whole bone marrow to obtain representative CFU-F counts [19]. A typical well within a six-well tissue culture plate has an available surface area of approximately 10 cm^2. In requiring an average of three or more CFU-F per well, the lower limit of detection of CFU-F frequency was established at roughly 30 or 10 CFU-F per million nucleated cells (0.003% or 0.001%), depending on the plating density used. Almost all patient samples (95 of 98) yielded an average of three or more CFU-F per well at one or both plating densities, suggesting the current approach adequately captures the prevalence of progenitor cells across our patient population. Although a small portion of patient samples (3 of 98) did not yield an average of three or more CFU-F per well at the higher plating density of 30×10^3 nucleated cells per cm^2, sporadic colonies were observed within all culture plates.

Previous studies have documented success in recovering viable nucleated cells from cryopreserved bone marrow [15,16,23,24]. However, the primary focus of these earlier studies was on the culture expansion of progenitor cells for clinical use and not the quantification of the initial progenitor cell content. Although there have been limited reports of unchanging CFU-F numbers between fresh and cryopreserved bone marrow, minimal data have been provided [15,17]. The nucleated cell viabilities measured within our cryopreserved BMAC samples (74% ± 9%) are similar to those previously reported [15]. Although a limited number of samples exhibited higher cell recoveries than expected following cryopreservation, BMAC is a heterogenous cell solution that can be viscous in nature, complicating efforts to transfer small, precise and well-mixed volumes. Given the high nucleated cell concentrations within the BMAC samples studied (10^8 cells per mL), unintentionally transferring a small percentage greater or less than the target volume could variably affect the final number of frozen cells.

Our current data reveal significant correlations between fresh and cryopreserved BMAC with respect to CFU-F frequency ($r = 0.87$) and mean colony area ($r = 0.49$), demonstrating the utility of cryopreserved BMAC in determining the prevalence of connective tissue progenitor cells. A small, albeit significant, pairwise difference in CFU-F frequency was observed (56.9 ± 34.8 vs. 50.8 ± 32.0 per million nucleated cells), suggesting that results obtained from cryopreserved samples may provide an underestimation of the true values. Yet not all cryopreserved BMAC samples produced fewer CFU-F than their fresh counterpart. Moreover, no significant pairwise differences were observed between fresh and cryopreserved BMAC with respect to mean colony area and density, as the data was more variable. Some BMAC samples produced larger, denser colonies after being frozen, but others did not.

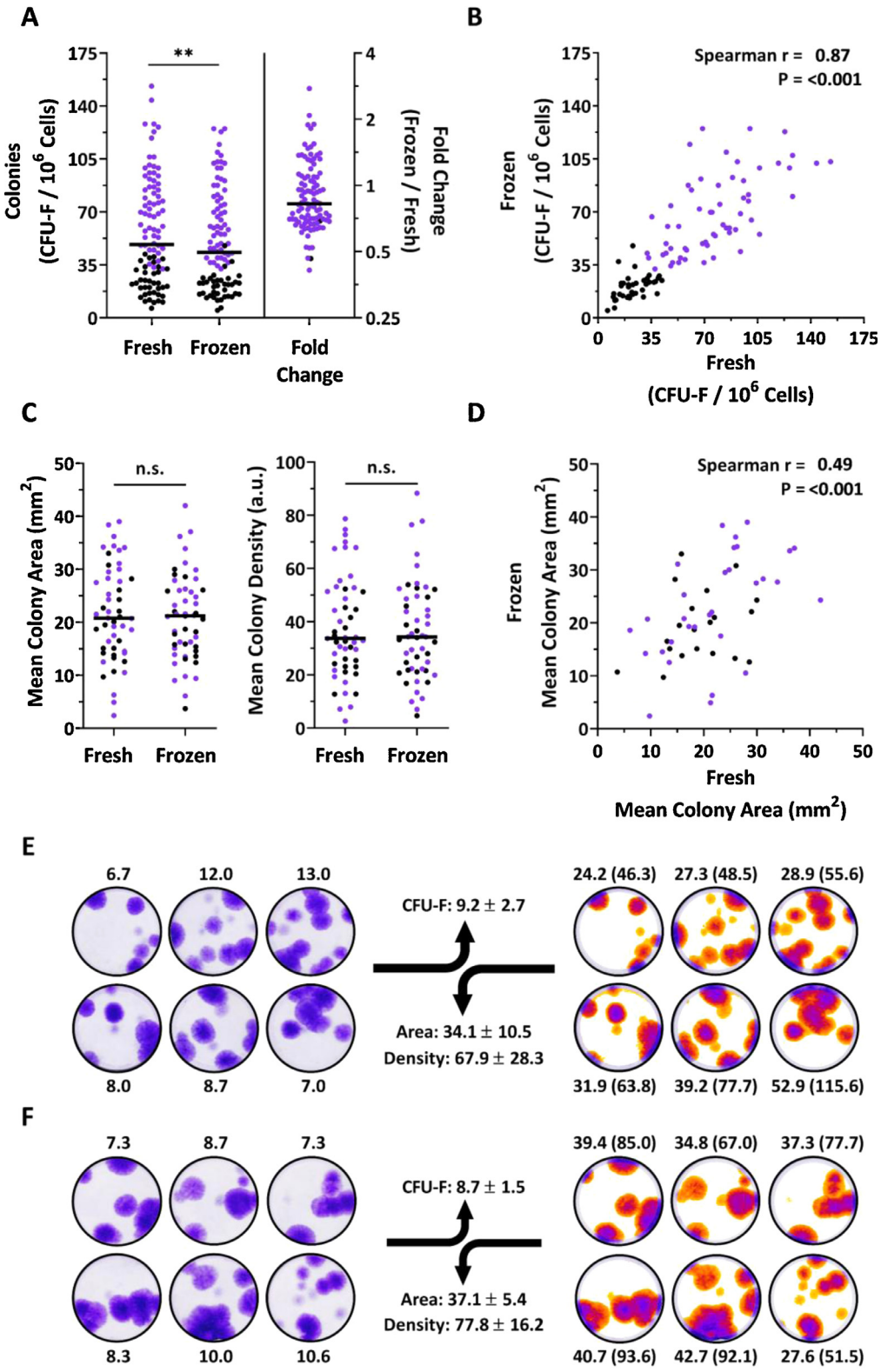

Figure 3. Cryopreserved BMAC performs similar to fresh BMAC in the CFU-F Assay. (A) CFU-F frequencies from individual donors (n = 98) counted within fresh and frozen BMAC samples and the resultant fold change. (B) Spearman rank order correlation between fresh and frozen BMAC with respect to CFU-F frequency. (C) Mean colony area and density quantified from high-resolution scans of assay plates obtained from a subset of donors (n = 52). (D) Spearman rank order correlation between fresh and frozen BMAC with respect to mean colony area. Violet data points are from BMAC samples plated at 10×10^3 nucleated cells per cm^2; (A and B, n = 60 of 98, C and D, n = 32 of 52); black data points are from BMAC samples plated at 30×10^3 nucleated cells per cm^2; (A and B, n = 38 of 98, C and D, n = 20 of 52); lines represent the median; Wilcoxon signed rank test $^{**}P < 0.001$ versus frozen BMAC. Representative scans and colony analysis of CFU-F within (E) fresh and (F) frozen BMAC samples from a single donor; average CFU-F counts from three independent observers are displayed above and below the wells on the left; mean colony area and density are displayed above and below the wells on the right; six-well plate averages (mean ± SD) are displayed in the center.

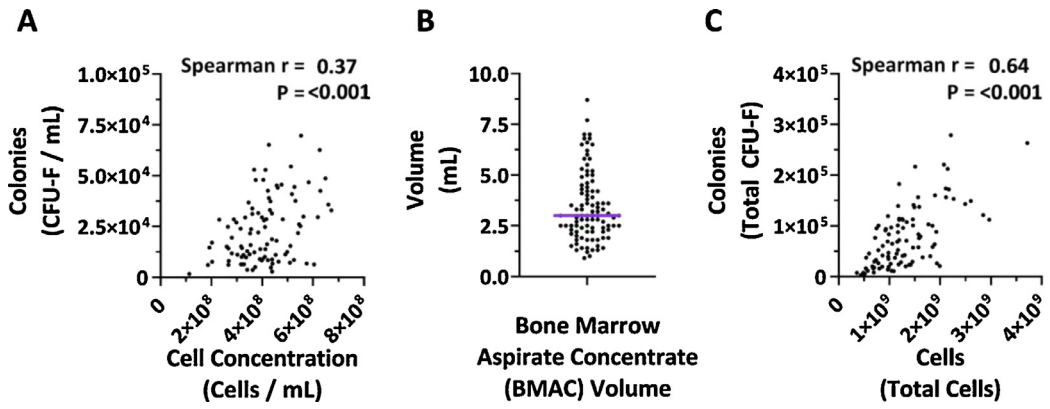

Figure 4. Colony formation is correlated with the total nucleated cell count. (A) Spearman rank order correlation between CFU-F concentration and nucleated cell concentration in fresh BMAC. (B) Volume of BMAC following laboratory processing; the line represents the median. (C) Spearman rank-order correlation between total CFU-F and total nucleated cells; (n = 98).

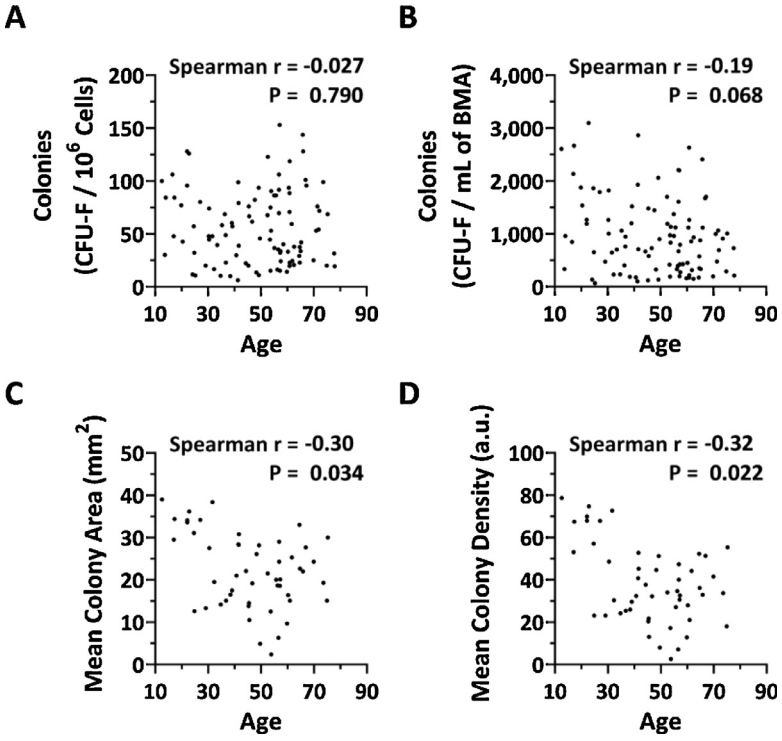

Figure 5. Colony formation, but not CFU-F frequency, is negatively correlated with patient age. Spearman rank-order correlations between age and (A) CFU-F frequency, (B) CFU-F when normalized to the volume of bone marrow aspirated, (C) mean colony area and (D) mean colony density in fresh BMAC (A and B, n = 98; C and D, n = 52).

Significant positive correlations were observed between the number of nucleated cells and CFU-F within both fresh and cryopreserved BMAC as well. Several groups have reported improved healing responses in patients treated with BMAC preparations containing higher concentrations of CFU-F [11,12]. Together, these data support our earlier findings of higher BMAC cell concentrations resulting in better pain outcomes for the treatment of knee osteoarthritis [25]. A linear relationship between CFU-F frequency and patient age was not observed, regardless of whether fresh or cryopreserved BMAC was used, as concluded in several studies [12,26,27]. Further, unlike other reports [11,28], no additional correlations with age were found when patient samples were separated by gender (data not shown). However, when accounting for the volume of bone marrow aspirated, a weak negative relationship between patient age and the number of CFU-F became apparent, although the correlation was not statistically significant. The presence of a negative correlation between CFU-F frequency and age has been described in more than one study [17,29,30]. Recently, age-related declines in CFU-F frequency, area and density within minimally cultured bone marrow aspirates have been reported, yet large donor-to-donor variation led the authors to conclude that age was not a reliable predictor of progenitor cell number [31]. Although our data failed to show a correlation between CFU-F frequency and age, significant declines were observed in both mean colony area and density, aligning well with reports of bone marrow progenitor cell frequencies remaining stable with age while their ability to proliferate declines [27].

Variation in CFU-F frequencies can arise intrinsically from patient-specific differences in bone marrow architecture and cellularity [32], whereas extrinsic sources of variation include the bone marrow aspiration site [33], technique [34] and volume [32], as well as the process used to concentrate the progenitor cells. A wide range of bone marrow volumes was aspirated from the patients included in the present study, resulting from the requirements of the different orthopedic procedures involved. To minimize hemodilution and maximize

the yield of progenitor cells, additional aspiration sites were used when obtaining larger BMA volumes [35]. Given the inherent variation in CFU-F frequency, a considerable number of BMAC samples may need to undergo analysis to establish evidence for a relationship between the prevalence of progenitor cells and patient outcomes. One advantage of using cryopreserved BMAC samples is the capability to analyze all samples of interest at once.

Multiple laboratory variables play an important role in colony formation. In addition to cell separation technique [21], plating density [20] and culture duration [22], other contributing factors including basal medium [7], serum [13], the addition of growth factors or other supplements [36], oxygen concentration [37], culture handling, and counting technique can affect overall CFU-F results. The ability to maintain consistency among these factors highlights another important advantage of performing retrospective batch analyses on cryopreserved BMAC samples. Efforts were taken to use controlled culture conditions and minimize sources of laboratory variation throughout our studies, and average colony counts from three independent observers were used to minimize bias. Nevertheless, manual enumeration of CFU-F remains imprecise and subjective at best [38]. Although high-resolution images were acquired and automatically analyzed for CFU-F area and intensity as an objective measure of colony formation, the reported measurements were averaged over an entire well and did not consider individual CFU-F characteristics [18]. More powerful analytical tools, capable of providing colony-specific metrics [38,39] and increasingly complex assays, such as those based on surface marker expression of connective tissue progenitors [28], could be used to better characterize BMAC before and after cryopreservation.

Another limitation of our current study is the relatively short period of time (<2 months) that BMAC samples were cryogenically stored before CFU-F analysis. There have been reports of bone marrow products being successfully cryopreserved for more than a decade without losing progenitor cell activity [40]. Others have reported significant reductions in mononuclear and cultured progenitor cell viability following extended periods of cryopreservation [16]. To address the question of how long-term cryopreservation of BMAC affects downstream CFU-F analyses, a limited subset of study samples was thawed a second time, approximately 10 months after initially being thawed. Following this longer period of cryopreservation, cell viability did not decline, and a strong correlation was maintained with respect to CFU-F frequency. However, further investigations are required to better understand how the duration of cryopreservation influences the ability of a progenitor cell to form a colony.

To our knowledge, this is the first report of a strong linear relationship between fresh and cryopreserved BMAC with respect to results of the CFU-F assay. Our present findings support the prospective cryogenic storage of patient BMAC samples before determination of CFU-F frequency, eliminating the need for timely on- or offsite laboratory analysis of fresh patient samples. Moreover, samples can be selectively removed from cryopreservation and retrospectively analyzed simultaneously to maintain consistency among the laboratory factors that can adversely affect the CFU-F assay. It is important to consider the inherent donor-to-donor variability with respect to the frequency of progenitor cells in BMAC therapies and understand that the proposed methodology should be applied to patient cohorts of ample size for elucidating dose-based efficacy in orthopedic regenerative medicine going forward. Future investigations will use the CFU-F frequencies obtained from cryopreserved BMAC samples to compare the prevalence of progenitor cells within this bone marrow–based orthobiologic therapy to patient outcomes.

Conclusion

The present study demonstrates cryopreserved BMAC samples to function as a suitable proxy to fresh BMAC samples for the starting material in the enumeration of connective tissue progenitor cells using the CFU-F assay. Owing to the large inter-donor variability in progenitor cell frequency, our recommendation is to directly plate BMAC at densities of 10×10^3 and 30×10^3 nucleated cells per cm^2, thereby preventing overt overgrowth, while consistently yielding individual colonies for counting following a 14-day growth period. The prospective cryopreservation of all patient BMAC samples allows for retrospective CFU-F analyses of clinically relevant or important samples for comparing progenitor cell frequency with patient outcomes.

Funding

This study was funded by Regenexx.

Conflicts of Interest

Patient samples were obtained from the Centeno-Schultz Clinic. DRB and ETA are members of the Research and Development team at Regenexx; NJS is the Chief Scientific Officer at Regenexx; CJC is the Chief Medical Officer at Regenexx, an intellectual property rights/patent holder with Regenexx and an owner of the Centeno-Schultz Clinic.

Author Contributions

DB: collection of data, data analysis and interpretation and manuscript writing; EA: collection of data, data analysis and interpretation; CC: conception and design, provision of study material and final approval; NS: conception and design, manuscript writing and final approval.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.04.091.

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