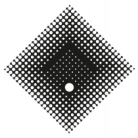
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Development and validation of a new method to isolate, expand, and differentiate circulating osteogenic precursor (COP) cells

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Development and validation of a new method to isolate, expand, and differentiate circulating osteogenic precursor (COP) cells

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ABSTRACT

Circulating osteogenic precursor (COP) cells are a population of progenitor cells in the peripheral blood with the capacity to form bone *in vitro* and *in vivo*. They have characteristics of the mesenchymal stem and progenitor pool found in the bone marrow; however, more recently, a population of COP cells has been identified with markers of the hematopoietic lineage such as CD45 and CD34. While this population has been associated with several bone pathologies, a lack of cell culture models and inconsistent characterization has limited mechanistic research into their behavior and physiology. In this study, we describe a method for the isolation of CD45+/CD34+/alkaline phosphatase (ALP) + COP cells *via* fluorescence-activated cell sorting, as well as their expansion and differentiation in culture. Hematopoietic COP cells are a discreet population within the monocyte fraction of the peripheral blood mononuclear cells, which form proliferative, fibroblastoid colonies in culture. Their expression of hematopoietic markers decreases with time in culture, but they express markers of osteogenesis and deposit calcium with differentiation. It is hoped that this will provide a standard for their isolation, for consistency in future research efforts, to allow for the translation of COP cells into clinical settings.

1. Introduction

As the use of stem cell and tissue engineering approaches becomes more widespread, there is a need for the identification and development of new cellular candidates to be applied clinically. The commonly used bone marrow mesenchymal stem and progenitor cells (MPCs) are highly proliferative, differentiate into a number of tissues, have low levels of immunogenicity, and secrete beneficial growth factors and immunomodulatory cytokines that induce tissue repair (Schaefer et al., 2016). While MPCs have wide-ranging clinical utility, they are also invasive to harvest, requiring bone marrow aspiration, and are known to cause immune reactions in some people (Galipeau and Sensébé, 2018). There are also concerns with pulmonary and vascular sequestration of the large mesenchymal stem cells (MSCs) when infused, with unclear endpoints (Furlani et al., 2009). In light of this, there is a need for alternative approaches to tissue engineering and stem cell therapeutics.

Circulating osteogenic precursor (COP) cells are a newly identified population of progenitors in the peripheral blood that have similar characteristics to MPCs (Feehan et al., 2021). COP cells have been shown to proliferate across multiple passages and to differentiate down mesodermal tissue lineages, much like their MPC counterparts. Indeed, initial studies showed that they were very similar, if not identical to the bone marrow cells (Zvaifler et al., 2000; Kuznetsov et al., 2001), and thus they were considered a surrogate population of MPCs that had been induced to circulate by some unknown stimulus. They were however believed to be extremely rare in the circulation (Kuznetsov et al., 2007), or indeed only present after pharmacological mobilization (Fernandez et al., 1997), or bone injury (Alm et al., 2010). While initially COP cells were shown to be very similar to the MPC, shortly after, a population of cells bearing the markers of the hematopoietic lineage was identified (Kuwana et al., 2003). MPCs are, by widely used criteria, unable to express the markers CD45, CD34, or CD14 (Dominici et al., 2006), however COP cells with these markers had the same capacity for multilineage mesenchymal differentiation (Kuwana et al., 2003). These hematopoietic COP cells have since been widely studied and offer several advantages for potential clinical utilization. They are present in steady numbers throughout the lifespan in healthy adults of both sexes (Gunawardene et al., 2017), and are associated with a number of pathological states of bone such as fracture (Eghbali-Fatourechi et al., 2005), heterotopic ossification (Suda et al., 2009), and osteoporosis

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(Feehan et al., 2021; Pirro et al., 2010). Despite increasing research into hematopoietic COP cells, there is still much to be explored to facilitate their extensive clinical utilization. Most of the evidence surrounding this cell population is observational in nature, due in part to the lack of an established tissue culture model. This is primarily because of the relative scarcity of COP cells in the circulation and widespread disagreement on the marker characterization across both hematopoietic and non-hematopoietic COP cell populations. Further, a number of the more commonly used markers of COP cells are intracellular products such as osteocalcin (OCN), which necessitate fixation and subsequently prohibit cell expansion in culture. To combat these issues, a consistent methodology for the isolation and expansion of COP cells is required to drive empirical *in vitro* and translational experiments.

To provide a consistent model for the evaluation of COP cells, this study aimed to describe and validate a methodology for their isolation and expansion in culture, as well as to identify changes in their expression of hematopoietic markers. It was hypothesized that hematopoietic COP cells would be identifiable by a discrete panel of markers, and that they would expand and undergo osteogenesis in culture.

2. Materials and methods

2.1. Setting

All procedures were performed in the PC2 laboratory facilities at the Australian Institute for Musculoskeletal Science (AIMSS), Western Health, Melbourne, Australia, under standard laboratory conditions, with aseptic technique.

2.2. Marker selection

To establish the optimal marker panel to identify COP cells in the peripheral blood, papers included in the two comprehensive literature reviews (Feehan et al., 2021; Feehan et al., 2019) were assessed to identify the cellular markers used to characterize COP cells. In order to select the markers that could be identified in donor blood, rather than after adherence and proliferation, only markers of fluid phase of circulating COP cells (*i.e.*, not cultured) were included. Once identified, common markers and combinations were assessed to determine the best panel that could be used for fluorescence-activated cell sorting (FACS).

2.3. Buffy coat sample purification

Buffy coat samples (n = 24) from healthy donors were acquired from the Australian Red Cross blood service (ARCBS) as a waste component of therapeutic red blood cell products. Samples were given without patient identifiers; however, the gender and date of birth were provided. Donors were aged between 18 and 75 were in good health and passed a medical history screening that excluded people with communicable, cardiovascular, and a number of other diseases, however, only donors from 20 to 40 years old were used in the analysis. The Western Health Human Research Ethics Committee issued a waiver of ethics review, as the donors consented to their samples being used for research at the time of donation.

The whole blood samples taken from the donors by the ARCBS were centrifuged without density gradient separation solution on site, and the buffy coats provided for research were contaminated with both a small number of red blood cells and a significant number of platelets. The buffy coats provided are calculated at 10% of the total volume taken, resulting in a 40–50 mL sample, taken from a whole blood donation of 450–500 mL, calculated on donor weight. To collect purified peripheral blood mononuclear cells (PBMCs), the buffy coats underwent a Ficoll density gradient centrifugation. The samples were diluted 2:1 with phosphate-buffered saline (PBS) supplemented with 5% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and layered on 12 mL of Ficoll solution (GE Healthcare Companies, USA) in 50 mL centrifuge tubes. This was centrifuged at 400 ×*g*, for 40 min with the deceleration set to the lowest level, and the buffy coat was removed from the interface of the Ficoll solution ensuring minimal contamination with plasma or Ficoll solution. The buffy coats were then diluted 10:1 with PBS and centrifuged at 100 ×*g* for 10 min, three times to remove contaminant platelets and Ficoll solution.

2.4. Immunofluorescent labelling

PBMCs were washed and suspended in 1 mL of PBS with 5% FBS, and incubated with FcR blocking reagent (BD Biosciences, USA) for 10 min at room temperature. The PBMCs were then incubated with the antibody cocktail described in Table 1 for 30 min at 4 °C in the dark, before being washed three times at 300 ×g for 5 min in PBS.

2.5. Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) isolation was performed on a BD FACSAria III (BD Biosciences, USA), with a four-laser configuration as described in Supplementary Table S1. A 70-micron nozzle was used to ensure the fastest sort time. Samples were collected on ice into recovery media consisting of low glucose, Dulbecco's modified eagle medium (DMEM) supplemented with 15% FBS, 100 I.U/mL penicillin, 100 μg/mL streptomycin, and 2.5 mM L-glutamine. The sorting was performed under aseptic conditions, with HEPA filtered air intake and 80% v/v ethanol sterilized flow lines. The sample acquisition chamber and flow cell were refrigerated at 4 °C, and 100 rpm tube agitation was used to prevent settling and aggregation of the cell suspension during sorting. Gating and sort strategies were performed on BD FACSDiva software Version 8.01 (BD Biosciences, USA). Single color and fluorescence minus one (FMO) control were used to control for fluorescence spread across channels. Due to individual differences in expression of markers and cell morphology between donors, and intra-day fluctuation in the instruments, gating was adjusted for each sample as required against donor-specific negative and FMO controls. Finally, 7-Aminoactinomycin D (7-AAD) was used as a viability marker, added 10 min prior to sorting.

2.6. Fibronectin matrix coating

Tissue culture flasks were prepared with a fibronectin coating to evaluate the impact of matrix factors on cell adhesion and proliferation. Matrix coating was performed on the same day of sample acquisition to control for degradation of unused plates. Lyophilized fibronectin obtained from human plasma (Sigma Aldrich, USA) was diluted in autoclaved sterile water and stored in working aliquots at −20 °C until required. Standard, sterile 25cm^2 plastic tissue culture flasks were coated with either 25 μg (1 μg/cm^2) or 75 μg (3 μg/cm^2) of fibronectin dissolved in the least volume of water. These were left to air dry in a biosafety cabinet for 1 h and were stored at 4 °C until required.

2.7. Cell culture and passaging

Isolated COP cells were cultured in low-glucose DMEM as described

Table 1
Fluorochrome antibody conjugates. FITC: Fluorescein isothiocyanate, APC: Allophycocyanin, BV421: Brilliant Violet 421, 7-AAD: 7-Aminoactinomycin D.

Antibody target	Fluorochrome	Manufacturer	Concentration
CD45	FITC	BD Biosciences	1:100 v/v
CD34	APC	BD Biosciences	1:100 v/v
Tissue non-specific alkaline phosphatase	BV421	BD Biosciences	1:100 v/v

above immediately after FACS. The cells were plated at a density of 0.6 $\times 10^5$ cells/cm^2 and incubated in a standard tissue culture incubator at 37 °C and 5% CO_2. Once confluent, cells were washed with PBS, then incubated with TripLE Express (Thermo Fisher, USA) cell dissociation agent for 5 min, aspirated, washed at 300 ×g for 5 min before being re-plated.

2.8. Osteogenic differentiation

At 70% confluence, the cells were cultured in osteogenic differentiation media (ODM) with the osteogenic supplements 10 mM β-glycerol phosphate (Sigma-Aldrich, St Louis, MO, USA, cat no. G9891), 0.5 mM ascorbic acid (Sigma-Aldrich, St Louis, MO, USA, cat no. 255564) and 0.1 μM dexamethasone (Sigma-Aldrich, St Louis, MO, USA, cat no. D4902) for up to three weeks.

2.9. Alkaline phosphatase and osteocalcin expression analysis

Expression of bone-specific alkaline phosphatase (ALP) and osteocalcin (OCN) was performed *via* flow cytometry. After seven and 21 days (for ALP and OCN, respectively) in osteogenic differentiation media, cells were harvested *via* TrypLE Express cell dissociation agent and permeabilized with the Cytofix/Cytoperm reagent kit (BD biosciences, Franklin Lakes, NJ, USA. Cat no. 554714). Cells were then stained with a pre-titrated concentration of Brilliant Violet 421 conjugated ALP antibody, or a PE-conjugated OCN antibody for 30 min at 4 °C in the dark. Then the cells were washed three times in FACS buffer by centrifugation at 300 ×*g* and analyzed on a BD FACSCanto II cytometer with a three-laser optics configuration (Supplementary Table S1). Seven individual samples each were used for the flow cytometric analysis of ALP and OCN.

2.10. Assessment of in vitro calcium deposition

After 21 days in osteogenic differentiation media, cells were stained using alizarin red (Sigma-Aldrich, St Louis, MO, USA), according to standard protocols (Stanford et al., 1995). Briefly, the medium was aspirated, and the cells were washed three times in PBS, then fixed for 30 min in 10% v/v formaldehyde in PBS. After fixation, formaldehyde was removed, and the cells were washed in 4.2 pH PBS three times, before being incubated with alizarin red solution in the dark for 15 min. Samples were then washed under tap water, then with PBS and rotation, before being visualized. After visualization, the cells were de-stained by incubation with 10% cetylpyridinium chloride (CPC) in 10 mM sodium phosphate for 30 min. The resulting samples were diluted and analyzed on a chemiluminescent plate reader for absorbance at 562 nm. Alizarin red assessment was performed on three unique donors, each plated in triplicate.

2.11. Quantitative real-time PCR

RNA from COP cells cultured in growth and differentiation media for 10 days were collected with QIAGEN miRNeasy Minikit (Hilden, Germany) as per manufacturer instructions for real-time PCR. PCR analysis of differentiation was performed on three unique donors, each plated in triplicate. RNA was quantitated *via* nanodrop spectrophotometer and quality control performed by Agilent bioanalyzer with RNA integrity number (RIN) >9 deemed acceptable for end analysis. The cDNA synthesis was performed with the Bio-Rad IScript cDNA synthesis kit (Bio-Rad, CA, USA), in an MJ Research PTC-100 thermal cycler per manufacturer instructions. Bio-Rad SSO advanced universal SYBR green supermix was used for PCR reactions. Validated PrimePCR primer pairs for detecting OSX and RUNX2 (Bio-Rad, CA, USA) were used, with GAPDH used as an endogenous control. These primers are validated with >95% transcription efficiency. Real-time qPCR was performed using a Bio-Rad CFX96 instrument. PCRs were performed on samples from three individual donors, each plated in triplicate, and the $2^{-\Delta\Delta Ct}$ method used to calculate fold regulation in the genes of interest compared to controls.

2.12. Morphological assessment with DAPI and phalloidin staining

To clearly visualize the morphology of COP cells in culture, co-staining of the nuclei with 4′,6-diamidino-2-phenylindole (DAPI) and the cytoskeletons with a phalloidin-FITC conjugate was performed. Cells were fixed in 10% formalin for 15 min, before being stained with phalloidin at a concentration of 1 μg:5 μL in PBS. The cells were incubated for 15 min in the dark at 4 °C, before being washed and stained with DAPI at a concentration of 1:4.7 for 2 min in the dark. The cells were then washed twice with PBS and imaged on a Canon/Nikon inverted fluorescence microscope with required optical filters.

2.13. Statistical analysis

All statistical analysis was performed using the IBM SPSS statistics package (v26), and data visualization with RStudio (v1.3.1093). Independent *t*-tests were used to identify differences between group means, with alpha level of significance set at 0.05, with two-tailed p values being reported.

3. Results

3.1. COP cell marker selection

Forty-eight studies of COP cells were identified (Zvaifler et al., 2000; Kuznetsov et al., 2001; Kuznetsov et al., 2007; Fernandez et al., 1997; Alm et al., 2010; Kuwana et al., 2003; Gunawardene et al., 2017; Eghbali-Fatourechi et al., 2005; Suda et al., 2009; Pirro et al., 2010; Al Saedi et al., 2018; Gunawardene et al., 2015; Eghbali-Fatourechi et al., 2007; Rattazzi et al., 2016; Pasqualini et al., 2019; Pirro et al., 2012; Pirro et al., 2011; Peris et al., 2013; Matsumoto et al., 2006; Kelly et al., 2018; Mifune et al., 2008; Egan et al., 2018; Egan et al., 2011; Takahashi et al., 2016; Otsuru et al., 2017; Otsuru et al., 2007; Otsuru et al., 2008; Manavalan et al., 2012; Rubin et al., 2014; Rubin et al., 2011; Ritz et al., 2014; Fadini et al., 2012; Fadini et al., 2011; Tang et al., 2019; Dalle Carbonare et al., 2017; Dalle Carbonare et al., 2009; Sicco et al., 2018; Undale et al., 2010; Wang et al., 2014; Lee et al., 2020; Kumagai et al., 2012; Kumagai et al., 2008; D'Amelio et al., 2010; Pal et al., 2011; Pal et al., 2010; Wan et al., 2006; Yang et al., 2011; Iwakura et al., 2013), which were divided into three major groups: those describing COP cells expressing hematopoietic markers (n = 25), those specifically not expressing those markers (n = 12), and those whose hematopoietic lineage status had not been studied (n = 11). Based on this, the hematopoietic lineage cells were chosen as the subject of the study.

In this population, most commonly used markers were OCN (n = 18), ALP (n = 15), CD34 (n = 13), and CD45 (n = 10) with Col1 (n = 6) and CD105 (n = 3). CXCR4, CD73, CD90, CD44, Stro1, CD146, osteonectin (ON), and osteopontin (OPN) less commonly used. The most commonly used combinations of markers were $CD34^+/OCN^+/ALP^+$ (n = 6), $CD45^+/OCN^+$, $CD34^+/OCN^+$, $CD45^+/Col1^+$, $CD34^+/CD146^+/OCN^+$ (n = 3 each), and $CD45^+/CD14^+/OCN^+/ALP^+$ (n = 2).

Based on these results, the hematopoietic COP cell population was chosen to be studied, and the marker panel consisting of CD45, CD34, and ALP was selected to sort the cells due to their frequency of use, and location on the cell membrane allowing for the isolation of live cells. CD34 and ALP were both the most common markers commonly used together in the literature, and CD45 was added to exclude the non-hematopoietic COP cells, to ensure homogeneity in the face of the documented $CD34^{low}$ cells in the MSC-like population (Dalle Carbonare et al., 2017; Dalle Carbonare et al., 2009). Markers such as the commonly used OCN are unsuitable for use in live-cell sorting due to their intracellular location, necessitating fixation of the cells, and

associated non-viability.

3.2. $CD34^+/CD45^+/ALP^+$ COP cells are a discreet population in the circulation, and also express OCN

The COP cell sorting strategy is demonstrated in Fig. 1. Cell viability (expressed by negative staining by 7-AAD) was consistently high (Fig. 1A), and PBMCs sorted were consistent with the typical forward scatter and side scatter characteristics of primary blood cell populations (Fig. 1B). COP cells were selected based on their expression of CD45, CD34, and ALP (Fig. 1C and D). The number of sorted COP cells was consistent with past studies (Gunawardene et al., 2017), making up between 0.4 and 1% of the PBMC population (Fig. 2E). As expected, the COP cells were located primarily within the monocyte/granulocyte population based on their morphology (Fig. 1F). Sorted CD34+/CD45+/ALP+ COP cells also express OCN on repeat flow cytometry, with approximately 89% of COP cells expressing OCN (Fig. 1G).

3.3. COP cells require a matrix substrate for effective attachment and expansion in vitro

Sorted COP cells were cultured on uncoated plastic tissue culture flasks in growth media as described above, as well as surfaces that were coated with fibronectin at 1 μg/cm^2 and 3 μg/cm^2. Fig. 2 shows the resulting expansion in each condition at 7 and 10 days in culture. The cells grown on standard tissue culture plastic adhered in smaller numbers and failed to expand at any time point. COP cells grown on both 1 μg/cm^2 and 3 μg/cm^2 rapidly adhered and began to form fibroblastoid colonies. At day 3, the COP cells on 3 μg/cm^2 of fibronectin appeared to have stronger proliferation than those on 1 μg/cm^2, however this had equilibrated by day 7. Once expanded, transfer onto standard tissue culture plastic equally supported attachment and proliferation, compared to fibronectin-coated plates. Among the spindle-shaped cells are cells with a broader round morphology. Phalloidin staining showed a non-uniform distribution of actin throughout the cells, with increased staining in clusters particularly at the polar ends of the cells with a spindle morphology, and at the periphery of the round cells potentially indicating leading edges.

3.4. COP cells lose expression of hematopoietic markers in culture

After 5 days in culture, the sorted COP cells demonstrated a significant decrease in the expression of CD45, falling by 76.25% ($p \leq 0.05$) from the expression level at the initial time of isolation. There was also a small (19%), but statistically significant decrease in CD34 expression at the same time point ($p \leq 0.05$) (Fig. 3).

3.5. COP cells undergo osteogenic differentiation in culture

After 7 days in ODM, COP cells demonstrated a 1.8-fold increase in ALP expression compared to control cells that remained in growth medium (GM) ($p = 0.003$) (Fig. 4A). After 21 days in differentiation medium, COP cells showed a small (1.1-fold), but statistically significant increase in OCN expression ($p \leq 0.05$) (Fig. 4B), and higher levels of calcium deposition determined by alizarin red staining, with 3.7-fold stronger staining ($p \leq 0.001$) (Fig. 4C). COP cells in ODM for 10 days showed a 6.7-fold increase in OSX expression ($p < 0.001$), and a 3.1-fold increase in RUNX2, though this was nonsignificant (Fig. 4D).

4. Discussion

This study is the first to describe and validate a method of isolating hematopoietic lineage progenitor cells with the capacity for osteogenesis from the blood of healthy adults. The field of COP cell research has been limited by inconsistency in the characterization and identification of specific COP cell populations. It is hoped that this study will provide a consistent methodology for the isolation and expansion of these cells, which can be used to guide future research in the field. This

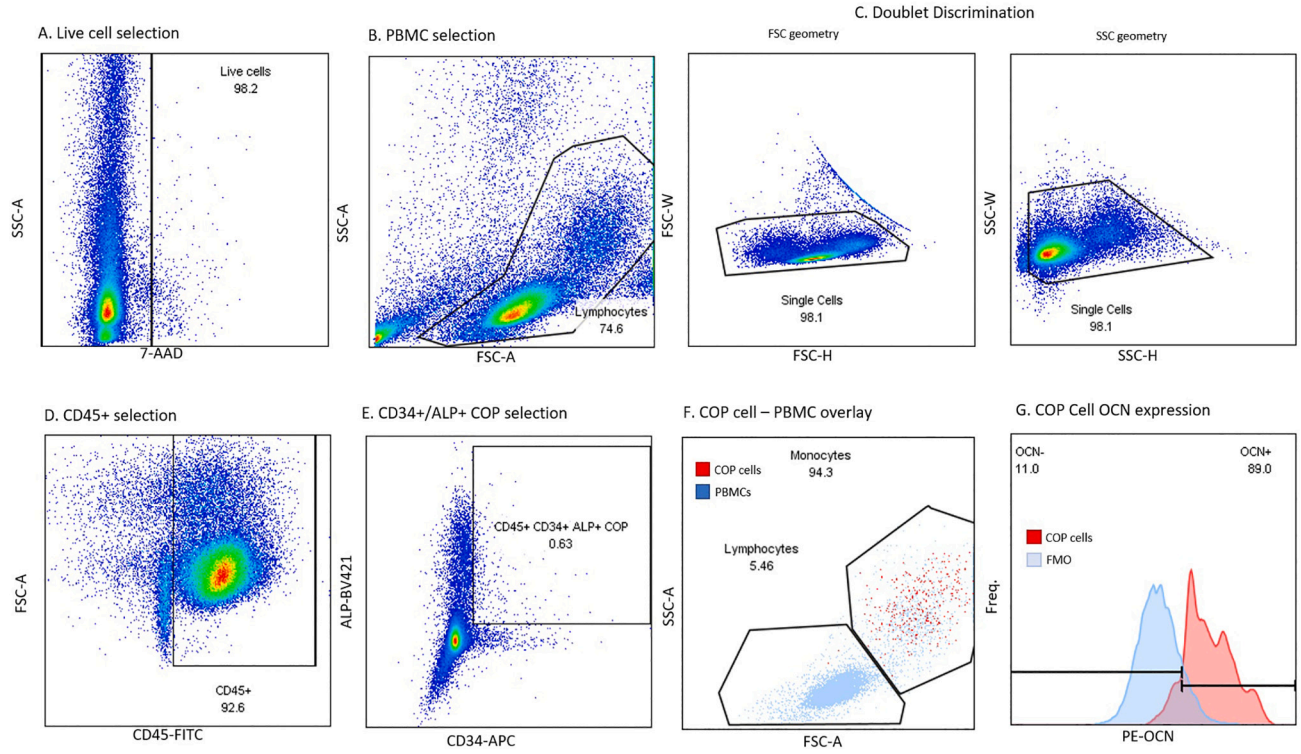

Fig. 1. Fluorescence-activated cell sorting strategy to isolate CD45+, CD34+, ALP+ COP cells. OCN: Osteocalcin, FMO: Fluorescence-Minus-One, FSC: Forward scatter, SSC: Side scatter, APC: Allophycocyanin, FITC: Fluorescein isothiocyanate, PBMC: peripheral blood mononuclear cells, ALP: Alkaline Phosphatase, 7-AAD: 7-aminoactinomycin D, BV421: Brilliant violet 421.

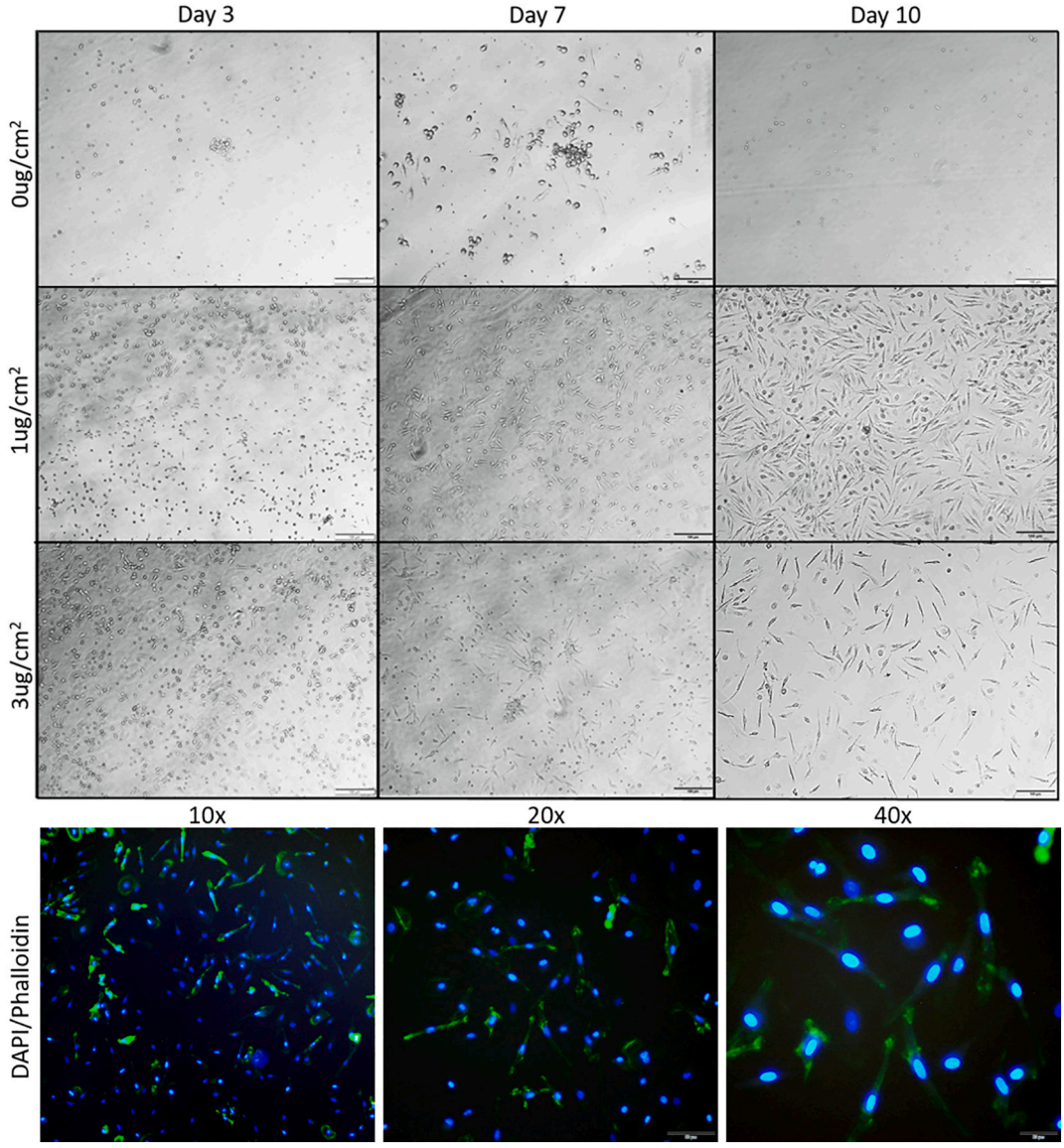

Fig. 2. *Top:* Cultured COP cells with different concentrations of fibronectin matrix coating. Images taken at 10×, at days 3, 7, and 10. *Bottom:* Cultured COP cells at 7 days, stained with DAPI (blue), highlighting the nuclei, and phalloidin (green) staining actin filaments in the cytoskeleton.

will offer several advantages to future research. The methodology is simple, and consistent across donors, with the only limitation being the volume of blood required to extract an adequate number of cells for subsequent culture. This is balanced by the comparative invasiveness and difficulty of stem cell harvesting from the bone marrow, lending COP cells strength as a candidate for stem cell therapies and diagnostics. The population of cells identified by FACS in this study is in line with the literature in terms of number, morphology, and marker expression, giving confidence in the authenticity of the isolated population. The number of hematopoietic (CD45+/OCN+) COP cells in the circulation has been shown to be consistent across age and gender in healthy patients, with a mean of 0.42% of the PBMCs (Gunawardene et al., 2017), and they are thought to reside among the monocyte population morphologically. The finding that COP cells require a matrix substrate to expand has interesting implications for researchers in the field and potentially accounts for the lack of reported cell culture models. Fibronectin is a key protein of the extracellular matrix, with a range of functions beyond structural support of local cells and tissues (Zollinger and Smith, 2017). Through its interactions with the membrane-spanning integrin receptors, fibronectin regulates a number of processes including cell migration, proliferation, and differentiation. It is possible that *in vivo* binding of fibronectin at the site of injury drives COP cell-mediated healing responses.

One of the key points of contention in COP cell research centers on their hematopoietic lineage status. As COP cells were originally thought to be a surrogate of the bone marrow MSCs, which by commonly accepted definition cannot express CD45, CD34, or CD14, the identification of mesenchymal progenitors bearing these markers led to a significant debate (Feehan et al., 2019). This study demonstrates the osteogenic potential of the hematopoietic population, supporting their status as progenitors, however, the relationship between them and the non-hematopoietic population still needs further exploration. In the present study, we demonstrate that hematopoietic COP cells lose CD45 expression in culture, which may have significant implications for future research. Most studies that have successfully grown COP cells in culture have shown them to be CD45 negative; however, this assessment is typically made after several days post-attachment. These studies typically place isolated PBMCs into a culture and assess the adherent proliferative population that forms (Zvaifler et al., 2000; Alm et al., 2010). Given the rapid loss of CD45 demonstrated in our study, this could lead

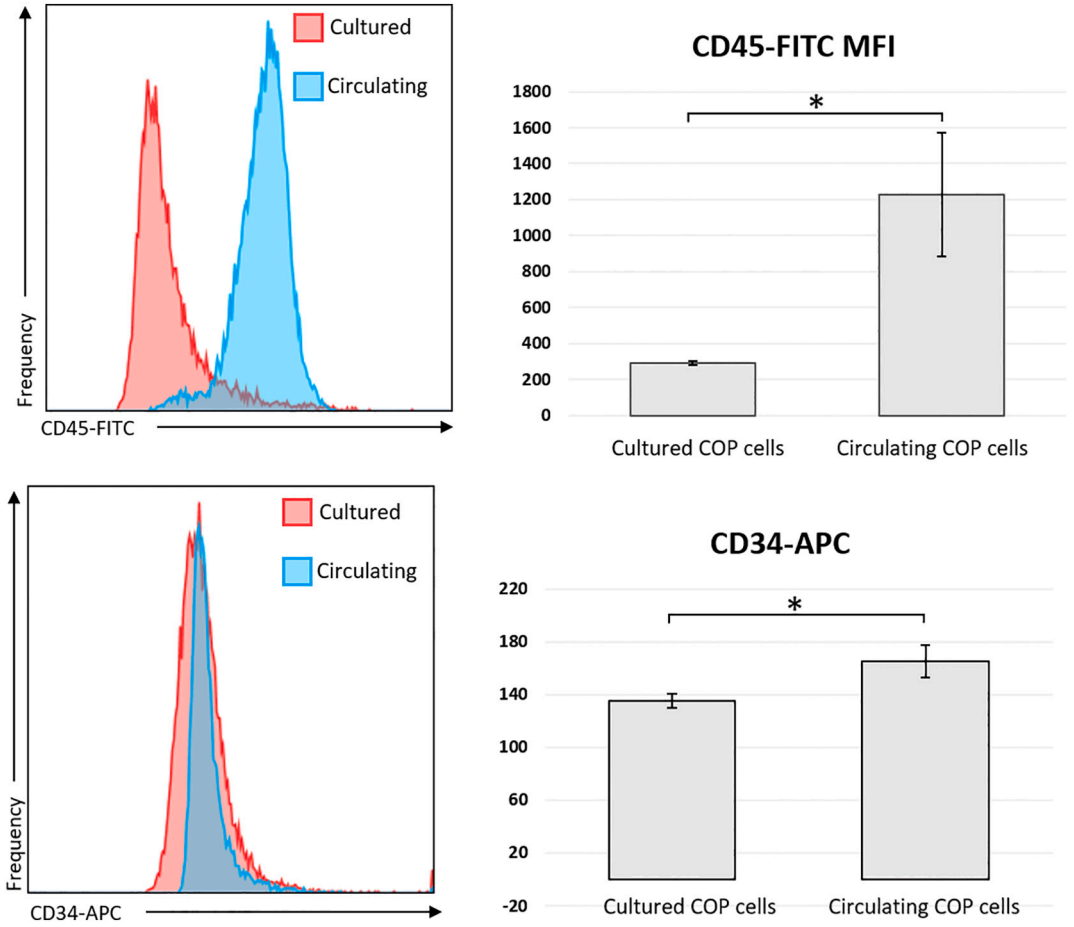

Fig. 3. COP cell expression of the hematopoietic markers, CD45 and CD34, after adherence. FITC: Fluorescein isothiocyanate, APC: Allophycocyanin, MFI: Median fluorescence intensity. Indicative histograms, bar charts descriptive of n = 12 donor samples.

to incorrect identification of COP cell lineage. This has been suggested in a novel triple transgenic mouse model which was able to identify cells which had expressed CD45 at any point of their differentiation, even if the marker was no longer present. The study showed that the hematopoietic cells did home to a fracture site and engraft within the callus, however, they did not appear to participate in bone formation directly as osteoblasts (Otsuru et al., 2017). The small decrease in CD34 expression demonstrated in our study is more difficult to interpret. Typically a marker of hematopoietic and vascular stem cells, CD34, is expressed by a range of tissues and is thought to be involved in cell adhesion (Sidney et al., 2014). Some authors have identified low expression of CD34 on MPCs (Copland et al., 2008), despite the original descriptions showing them as CD34- (Dominici et al., 2006). However, the current understanding of the bone marrow stem cells describes a heterogeneous population of progenitors with a range of lineages and functions (Karsenty, 2017). While our study identified a decrease in CD34 expression, low expression of CD34 was still present after attachment, which is consistent with previous studies (Dalle Carbonare et al., 2017; Dalle Carbonare et al., 2009), however, other studies have shown no expression of CD34 on COP cells (Zvaifler et al., 2000; Kuznetsov et al., 2001; Kuznetsov et al., 2007; Fernandez et al., 1997). While these findings offer some insight and explanation for the heterogeneous populations found in COP cell studies, full characterization of both the hematopoietic and non-hematopoietic COP cell populations at the initial isolation and after adherence in culture is required. This will allow for understanding of the identity of specific COP cell subsets, their relationships with one another, as well as their potential biological role.

While the rarer, non-hematopoietic COP population has been shown to form bone *in vitro*, studies conclusively showing osteogenesis, and more specifically, osteoblast differentiation in the hematopoietic lineage cells are lacking due to an absence of culture models. In our study there was a significant increase in ALP expression after seven days and effective calcium deposition after three weeks in osteogenic differentiation medium. They also showed an increase in expression of the critical osteogenic transcription factors OSX and RUNX2, further supporting their capacity for osteogenesis. This resonates with findings in animal models of hematopoietic COP cells, where their implantation into mice on bone morphogenetic protein 2 (BMP-2)-coated pellets was shown to generate new osteoid (Suda et al., 2009; Otsuru et al., 2017). There was also a small, but statistically significant increase in the expression of OCN after three weeks in ODM, however this was smaller than may have been expected given the associated findings of calcium deposition. While OCN has systemic metabolic effects (Karsenty, 2017); it does not play a direct role in calcium deposition, and its role in COP physiology is still unclear. With research into the pathways by which OCN is secreted still ongoing, it is possible that an unidentified signaling compound released as a part of the bone remodeling process triggers COP differentiation and expression of the hormone. These findings provide some evidence to support COP cells as an osteoblastic progenitor, but more research to characterize their differentiation is required. Whether COP cells terminally differentiate into osteoblasts, or whether they have a secondary mechanism of mineralization is still not well established. Cells with an osteoblastic phenotype have been demonstrated in settings of vascular calcification (Rajamannan et al., 2003), which occurs independently of genuine osteoblasts – COP cells could potential be a mediator of these changes.

The pre-circulation lineage of COP cells is still unknown, with a specific bone marrow precursor not identified. The hematopoietic

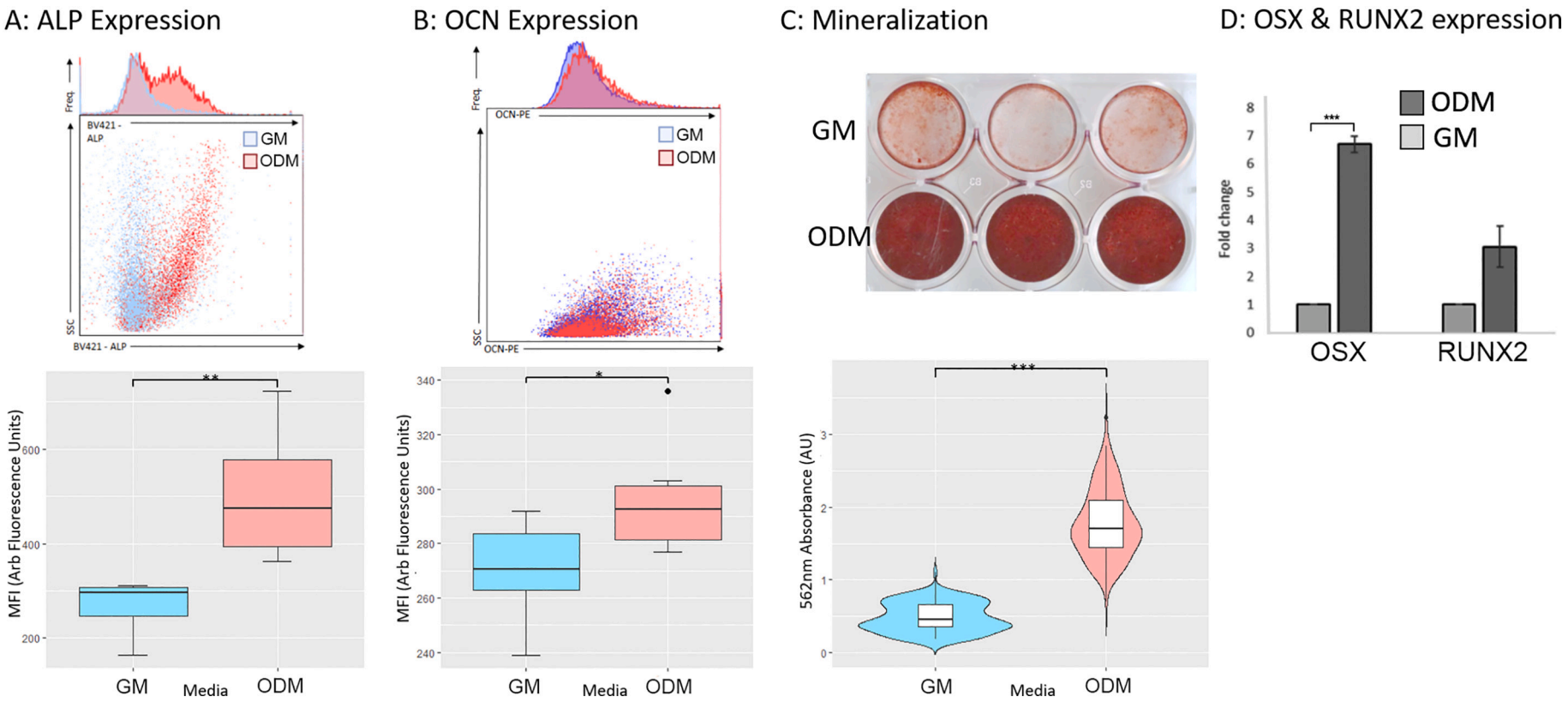

Fig. 4. Osteogenic activity of differentiated COP cells. All performed as 3 technical replicates each from 3 individual donors. *A:* COP cell expression of ALP after 7 days in culture. *B:* COP cell expression of OCN after 21 days in culture. *C:* Alizarin red staining of deposited calcium after 21 days in culture. ALP: Alkaline phosphatase, OCN: Osteocalcin, GM: Growth media, ODM: Osteogenic differentiation media, BV421: Brilliant Violet 421, PE: Phycoerythrin. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

lineage of COP cells suggests that the hematopoietic stem cell (HSC) could be a potential precursor. The HSC has been shown to be able to act as an osteoblast precursor, reconstituting the osteoblast pool in mice after transplantation (Olmsted-Davis et al., 2003), and also influence the behavior of MSCs in the bone marrow niche (Jung et al., 2008), potentially supporting this hypothesis. Indeed, the first report of circulating mesenchymal progenitors came from a study of HSC mobilization techniques in breast cancer patients (Fernandez et al., 1997); however, these were characterized as non-hematopoietic. Lineage studies to identify the relationships between these different progenitor populations are required to fully understand the *in vivo* functioning of these cells in states of bone healing and remodeling.

Although these results represent an important step in understanding COP cell physiology, there remain a number of unknowns. Future studies should aim to compare the hematopoietic and non-hematopoietic populations of COP cells, to identify inter-related behaviors and potential roles, while also confirming their dual existence. Studies should also perform a side-by-side comparison of COP cells and MPCs to evaluate factors such as immunogenicity and factor secretion to allow an understanding of their potential for clinical utilization. Whether COP cells are capable of multi-lineage differentiation is still incompletely known, particularly in the hematopoietic subset. Full mesenchymal lineage differentiation should be performed to identify whether they have limited osteogenic lineage or are indeed multipotent progenitors.

5. Conclusion

This study describes a means of isolating hematopoietic COP cells *via* FACS and validating their osteogenic potential. CD45+/CD34+/ALP+ COP cells are readily acquired from the blood in healthy individuals, but lose CD45 expression on tissue culture. COP cells may be a candidate for stem cell therapies and tissue engineering due to their capacity for differentiation and proliferation and ease of harvesting and isolation. Future studies should evaluate other details of their behavior *in vitro*, to identify their true clinical utility.

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Data availability statement

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

CRediT authorship contribution statement

Study design: JF, KN, CA, GD. Study conduct and experiments: JF. Data collection: JF. Data analysis: JF, KN, CA, GD. Data interpretation: JF, KN, CA, GD. Drafting manuscript: JF. Revising manuscript content: JF, KN, CA, GD. Approving final version of manuscript: JF, KN, CA, GD. GD takes responsibility for the integrity of the data analysis.

Declaration of competing interest

No conflict of interest to declare.

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