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Chondrogenic potential of blood-acquired mesenchymal progenitor cells☆

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Summary *Introduction:* The associated morbidity from the acquisition of mesenchymal stem cells (MSC) from the bone marrow has led to the investigation of alternative stem cell sources. We propose that such cells may be isolated from non-mobilised blood and demonstrate their differentiation into a chondrocytic lineage. This safe and abundant source of cells may be useful for tissue engineering cartilage.
Method: Peripheral blood mononuclear cells (PBMC) were isolated from healthy adults and cultured in RPMI medium supplemented with serum. The non-adherent and adherent cells were analysed for cell surface marker expression of CD14, CD34, CD133, CD105 and CD45 by flow cytometry. Adherent cells were also cultured on glass slides in chondrogenic media and analysed for the expression of collagen I and II on day 14 of culture.
Results: The adherent cells were fibroblastic in morphology and were confluent on day 14. The non-adherent and adherent cell populations were shown to have distinct profiles by flow cytometry. The adherent cells were positive for CD105 and CD14 and also expressed collagen I and II precursors when cultured in chondrogenic media.
Conclusion: Blood-acquired mesenchymal progenitor cells (BMPCs) can be isolated from non-mobilised blood. These unique cells are $CD105^+$ and $CD14^+$ and have chondrogenic differentiation capacity. BMPC may provide a potential source of MPC for tissue engineering applications.

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Cartilage has a poor regenerative capacity and its replacement tissue, fibrocartilage, is mechanically inferior. It plays an important role in joints (hyaline or articular cartilage) and for structural features (elastic cartilage) such as in the nasal septum. Chondrocytes are highly specialised cells which secrete extra-cellular matrix (ECM) proteins. The ECM of healthy hyaline cartilage consists of collagen II (approximately 90–95%), with lesser amounts of collagen types VI, IX, X, XI and also glycosaminoglycans (GAGs). The ECM of fibrocartilage consists of a greater proportion of collagen I than collagen II.

The mainstay of treatment for articular cartilage reconstruction is arthrodesis and arthroplasty, although various approaches to encourage cartilage regeneration have been attempted.[1,2] Current synthetic implants harbour problems, principally infection, rejection, longevity and unsatisfactory cosmesis.[3] Despite recent advances in tissue engineering, vascularisation of the tissue remains problematic.[4,5] Advances in tissue engineering have been limited by the requirement for most tissues to be vascularised. The relatively avascular nature of cartilage therefore makes it an ideal target for tissue engineering.[6] Chondrocytes are hard to isolate in humans, replicate slowly and are prone to phenotypic de-differentiation in culture.[7,8] This may be further affected by donor age and health status.[9,10] Hence, tissue engineering based on these differentiated cells is unlikely to prove successful, and has fuelled studies into the use of stem cells in the tissue engineering of cartilage.

The rapid evolution of stem cell research has been an important catalyst in tissue engineering. The unlimited replication capacity and multipotency of these cells undoubtedly elevate their value above that of primary differentiated cells as an application for tissue regeneration. Previous concepts of adult stem cells possessing predetermined differentiation potential have been found to be too regimented.[11] In addition to these expected pathways of differentiation (i.e. mesenchymal stem cells (MSC) differentiating into mesenchymal lineages and haemopoietic stem cells(HSC) differentiating into haemopoietic lineages), there have been studies showing HSC differentiating into hepatic oval cells, MSC differentiating into neural cells and neural stem cells differentiating into haemopoietic progenitor cells.[12–14]

Stem cells of the bone marrow (haemopoietic and mesenchymal) are the most commonly studied adult cells.[15,16] The isolation of MSC from the bone marrow is well documented, but remains a problematic cell source. Bone marrow aspiration is a painful procedure and MSC harvest is low (0.001–0.01%).[17] Alternative sources of MSC have been found in adipose tissue, muscle, trabecular bone, synovium, deciduous teeth and blood.[11]

The evidence that MSC exist in the peripheral blood is inconsistent. This may be due to the variety of methodologies used to isolate, purify and characterise these cells.[18] HSC markers are widely accepted as being $CD34^+$ and $CD45^+$.[19] CD34 is a cell surface glycoprotein involved in cell–cell adhesion and CD45 is a signalling molecule which is involved in the regulation of cell processes, such as cell growth and differentiation. There are no specific cell surface markers of MSC to date, but SH3, SH4, CD166 and CD105 are considered important markers of bone-marrow-derived MSC.[20,21] CD105 is of particular importance in chondrogenesis because it is part of the transforming growth factor-β1 complex.

Wexler and colleagues were unable to isolate MSC from the peripheral blood of healthy individuals.[22] This was determined by characterisation of haemopoietic surface markers on the isolated cell population (CD45 and CD14) and their non-mesenchymal phenotype. However, it has since been demonstrated that $CD14^+$ monocytes from healthy humans exhibit mesenchymal differentiation capacity.[23] Zvaifler and colleagues found that non-mobilised human blood possessed mesenchymal potential irrespective of the elimination of $CD34^+$, $CD3^+$ or $CD14^+$cells.[24]

It has also been proposed that circulating mesenchymal precursors may be present in pathological conditions. Fernandez isolated stromal cells from the blood of breast-cancer patients given granulocyte–monocyte colony stimulating factor (GM-CSF) following chemotherapy.[25] However, Lazarus and colleagues failed to demonstrate their presence in the mobilised peripheral blood of breast-cancer patients in a chemotherapy treatment regime.[26] A recent paper demonstrated the isolation of MSC in growth-factor-mobilised blood using $CD133^+$ cell selection.[27] CD133 has been strongly associated with HSC and its co-expression with CD34 has an important role in angiogenesis.[28–30] This finding therefore supports the growing theory that stem cells exhibit plasticity outside the categories of HSC and MSC. It is more accurate to use the term mesenchymal progenitor cells (MPCs) to describe cells that can differentiate into mesenchymal lineages, because their origin is not always clear.

The aim of this study is to further determine whether circulating MPCs are found in the blood of normal, healthy individuals and to demonstrate the chondrogenic potential of these cells.

Materials and methods

Cell isolation

Peripheral blood samples were taken from 10 consenting volunteers (aged 20–32 years) using a sterile heparinised vacutainer system. The blood was carefully layered over warm Lymphoprep® at a ratio of 2:1 (blood: Lymphoprep). Tubes were centrifuged at (800 g_{max}) for 25 min at room temperature, without break. Following centrifugation, the plasma layer was removed and stored at −20 °C for use in cell culture media (CCM). The buffy coat layer was removed with a 3 ml sterile plastic pipette (Falcon; BD Biosciences, Oxford, UK) and washed with sterile phosphate buffered saline (PBS) (Gibco, Biocult, Glasgow, Scotland). Tubes were centrifuged at 25 °C for 15 min at 600 g_{max}, followed by a second wash at 400 g_{max} for 10 min. Supernatant was removed and the peripheral blood mononuclear cells (PBMC) were counted with a haemocytometer using trypan blue exclusion.

Cell culture

The CCM used was RPMI-1640 glutamax (Sigma–Aldrich Company Ltd., Gillingham, Dorset, UK) supplemented with

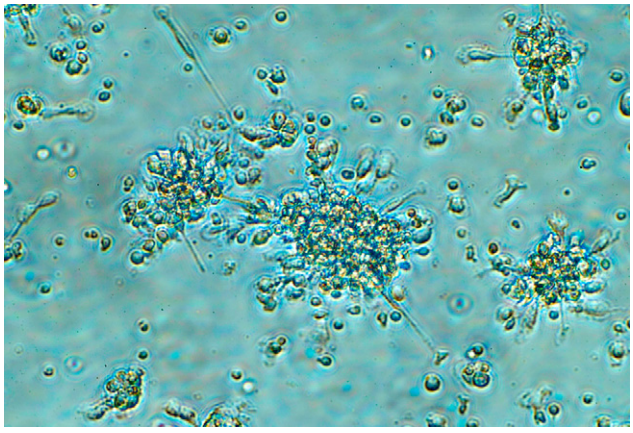

Figure 1 Phase contrast microscopy of BMPC at day 7 in culture (Magnification ×20).

10% v/v foetal bovine serum (FBS) (Sigma), 10% human serum (from blood samples), 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Gibco, Biocult, Glasgow, Scotland). PBMC were plated in T25 (Falcon; BD Biosciences, Oxford, UK) flasks at 10^6 cells ml^{-1} and placed in an incubator at 37 °C with 5%CO_2. On day 3 of culture, the non-adherent cells were collected and, following two gentle washes in PBS, the flasks replaced with new CCM. The CCM was changed every 3 days. On day 14, the adherent cells were collected using trypsin-like substance and counted using trypan blue exclusion and a haemocytometer. All adherent and non-adherent samples were re-suspended in 10% DMSO in FBS and transferred to liquid nitrogen until required.

Flow cytometric analysis

Following rapid thawing into cold CCM, re-suspended samples were aliquoted into sterile, polystyrene FACS tubes (Becton Dickinson) and centrifuged (600_{gmax}, 5 min, 4 °C). The anti-human antibodies CD133-PE (Miltenyi Biotec Ltd., Surrey, UK), CD105-APC (eBioscience, Ltd., Hatfield, UK), CD34-FITC, CD45-PERCP and CD14-PE (Becton Dickinson) were used to evaluate cell surface markers on the non-adherent and adherent cell samples. The anti-mouse antibodies IgG1-PE and IgG1-FITC (BD Pharmingen) were used for isotope control analysis. To each tube, 20 μl of the appropriate antibody cocktail was added and incubated at 4 °C in the dark for 15 min. Cells were washed in 1 ml of FACS buffer (PBS/1%FCS/0.2%Az) and centrifuged (600 g_{max}, 5 min, 4 °C). Supernatant was removed and cells re-suspended in 1% paraformaldehyde for FACS analysis. Samples were analysed on a FACSCalibur (Becton Dickinson). Viable lymphocytes were gated according to 0° and 90° light scatter profiles and data was analysed using FlowJo software (Tree Star Inc. OR, USA). The isotype controls were used to gate on the negative population and thus determine the true expression of cell surface markers.

Chondrogenesis

Adherent cells from PBMC isolation that had been cultured for at least 3 weeks were seeded at 10^6 per well in a 24-well plate containing glass slides. The CCM was removed at 48 h and replaced with 1 ml per well of chondrogenic media. This was a serum-free medium comprising RPMI-1640 glutamax (Sigma), 50 μl ml^{-1} ascorbate-2-phosphate (Fluka), Premix ITS$^+$ (BD Biosciences), 10 ng ml^{-1} TGF-β3 (Sigma) and 100 nM dexamethasone (Sigma). The chondrogenic media was changed every 3 days for 14 days.

Immunostaining

At 14 days, the chondroinduced cells were washed twice in 10 mM PBS and fixed with 4% paraformaldehyde for 20 min. The slides were then washed three times in PBS and incubated with 4% fat-free milk in PBS for 20—30 min. This blocking solution was then removed, followed by a wash in PBS. The primary polyclonal antisera, goat anti-human collagen I IgG (C18) and goat anti-human collagen II IgG (N19) were added at a concentration of 1:300 with 1.5% fat-free milk and incubated at room temperature for 60 min.

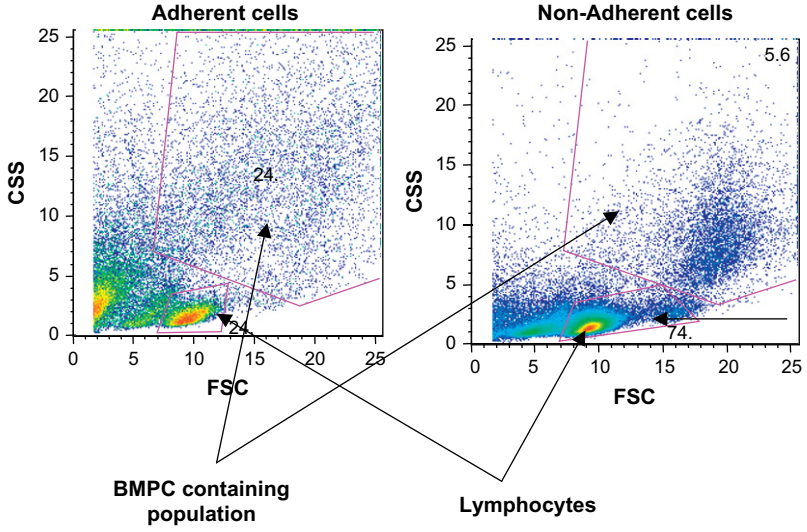

Figure 2 Scatter graphs showing the distribution of cells with respect to gate for BMPC and non-adherent cells.

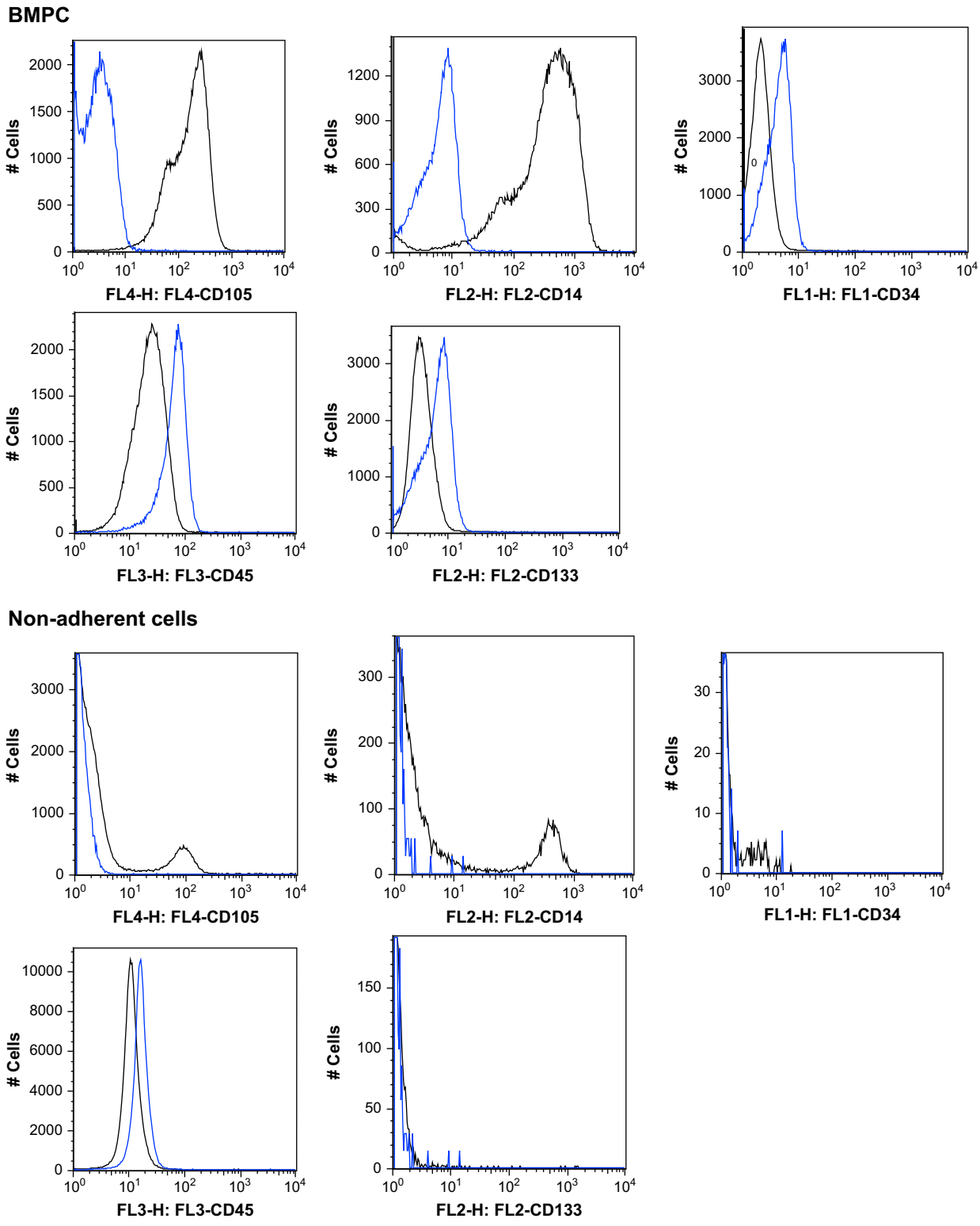

Figure 3 Histograms of cell surface marker expression in BMPC and non-adherent cells. Ig-G controls are shown in blue. BMPC are CD 105^{+} $CD14^{+}$.

These antibodies are recommended for the detection of collagen precursors and mature collagen. These samples were washed thrice with PBS and a donkey anti-goat IgG (sc-2024) secondary antibody conjugated to FITC in 1.5% fat-free milk in PBS was added. The slides were incubated for 45 min and then washed in PBS. The slides were then placed in propidium iodide at 1:1000 in 10 mM PBS to visualise the cellular DNA. The samples were mounted and viewed with a confocal microscope (Radiance 2100 (Bio-rad)). All antibodies were obtained from Santa Cruz

Biotechnology. The control slides were incubated with the secondary antibody only.

Statistical analysis

Percentages of cells expressing the various markers within the BMPCs and non-adherent gate were analysed using one-way analysis of variance (ANOVA) with post-hoc Bonferroni comparison.

Results

Cell culture

A small proportion of PBMC adhered to tissue culture flasks following day 3 of culture. This cell population contained cells of a round morphology. On day 7, defined clusters of fibroblastic cells supported by round stromal-like cells typically formed (Figure 1). On day 14, the majority of the cell population displayed a fibroblastic morphology and the flasks were 90–100% confluent. It is these cells which we refer to as BMPC. BMPC were passaged successfully at least 10 times in 8 of the 10 samples.

Surface protein expression of BMPC

Flow cytometry was used to assess the surface protein expression of BMPC and the non-adherent cell population. The non-adherent cells and BMPC were easily gated as distinct populations (Figure 2), therefore these appropriate gates were compared. The BMPCs were strongly positive for CD105 and CD14 and negative for CD34, CD45 and CD133 (Figure 3). CD105 and CD14 expression was significantly greater in BMPCs than in non-adherent cells ($p < 0.001$). There was no significant difference between CD34, CD133 and CD45 expression in either group (Table 1).

Immunostaining

All specimens showed evidence of the nuclei which stained red under the confocal microscope. The TGF-β3-treated BMPCs demonstrated intracellular localisation of both collagen I and collagen II precursors, which stained green (Figure 4). The phenotypes of the undifferentiated and TGF-β3-treated BMPC were similarly spindle shaped, but the latter were sparsely covering the slides. There was evidence of ECM in the undifferentiated BMPC when viewed under light microscopy, but this was not visible in the TGF-β3-treated BMPC. Neither cell populations showed evidence of collagen I or II extra-cellularly.

Table 1 Expression of cell surface markers in BMPC and non-adherent cell populations. Data are mean and standard deviation.

Antigen	Mean % positive cells (±SD)	
	BMPC ($n = 10$)	Non-Adherent Cells ($n = 5$)
CD34	2.25 (2.61)	0.23 (0.05)
CD133	1.54 (1.71)	0.07 (0.46)
CD45	80.47 (16.07)	94.17(1.70)
CD14	78.01 (32.04)	4.08 (3.33)
CD105	97.04 (2.59)	1.99(1.11)

Discussion

This study identifies a population of cells from PBMC of non-mobilised blood which possesses a chondrogenic differentiation capacity. This population is positive for CD105 and CD14, but negative for the characteristic HSC surface marker profile (CD34, CD133 and CD45).

The high expression of CD105 observed in BMPC strongly supports their mesenchymal potential. Majumdar and colleagues used magnetic beads to select $CD105^+$ cells from the bone marrow.[31] These cells were encapsulated in alginate and given chondrogenic media containing TGF-β3. Collagen II expression was detected by reverse transcriptase polymerase chain reaction (RT-PCR) and immunostaining following 21 days of such culture.

A subset of $CD14^+$ monocytes isolated from PBMC have been previously shown to possess mesenchymal capacity, but these cells were also $CD45^+$, CD 34^+ and type I collagen positive.[23] The acquisition of this cell population may be due to the difference in cell culture technique. For example, this group used fibronectin-coated plates and cultured their cells in Dulbecco's modified Eagle medium (DMEM), with 10% FBS at a density of 2×10^6cells ml^{-1}.

External factors inducing chondrogenesis of stem cells are well documented.[17,32] We demonstrated the presence of collagen I and II precursors in BMPC samples given a serum-free chondrogenic medium containing TGF-β3. Barry and colleagues found that the ECM of MSC cultured in chondrogenic media with TGF-β3 contained more GAGs and collagen II than those cultured with TGF-β1.[33] Other growth factors have been implicated in enhancing chondrogenesis of MSC, such as insulin-like growth factor-1 and fibroblast growth factor-2.[34,35] These will be investigated in light of the encouraging results of this preliminary study.

Collagen I and II precursors were found intracellularly in these chondro-induced cells. We considered that the investigation of the presence of precursors of collagen I and II would be more appropriate for this preliminary study than extra-cellular collagens because the duration of culture in chondrogenic media was too short for BMPC to establish a cartilaginous matrix. This study provides encouraging evidence that such protein synthesis can be induced; therefore, further work into the distribution and proportional output of GAGs and collagens I and II into an ECM is warranted here.

The morphology of the BMPC given chondrogenic media cells did not assume the typical round morphology of chondrocytes. De-differentiation of chondrocytes is known to occur in monolayer culture; therefore, using micro-mass culture or cell seeding into a 3D matrix may improve chondrogenesis.[7] It is also possible that the duration of culture in the chondrogenic media was too short for BMPC to establish a cartilaginous matrix. Using antibodies which detect precursors of collagen I and II enabled investigation into the early synthesis of these important proteins.

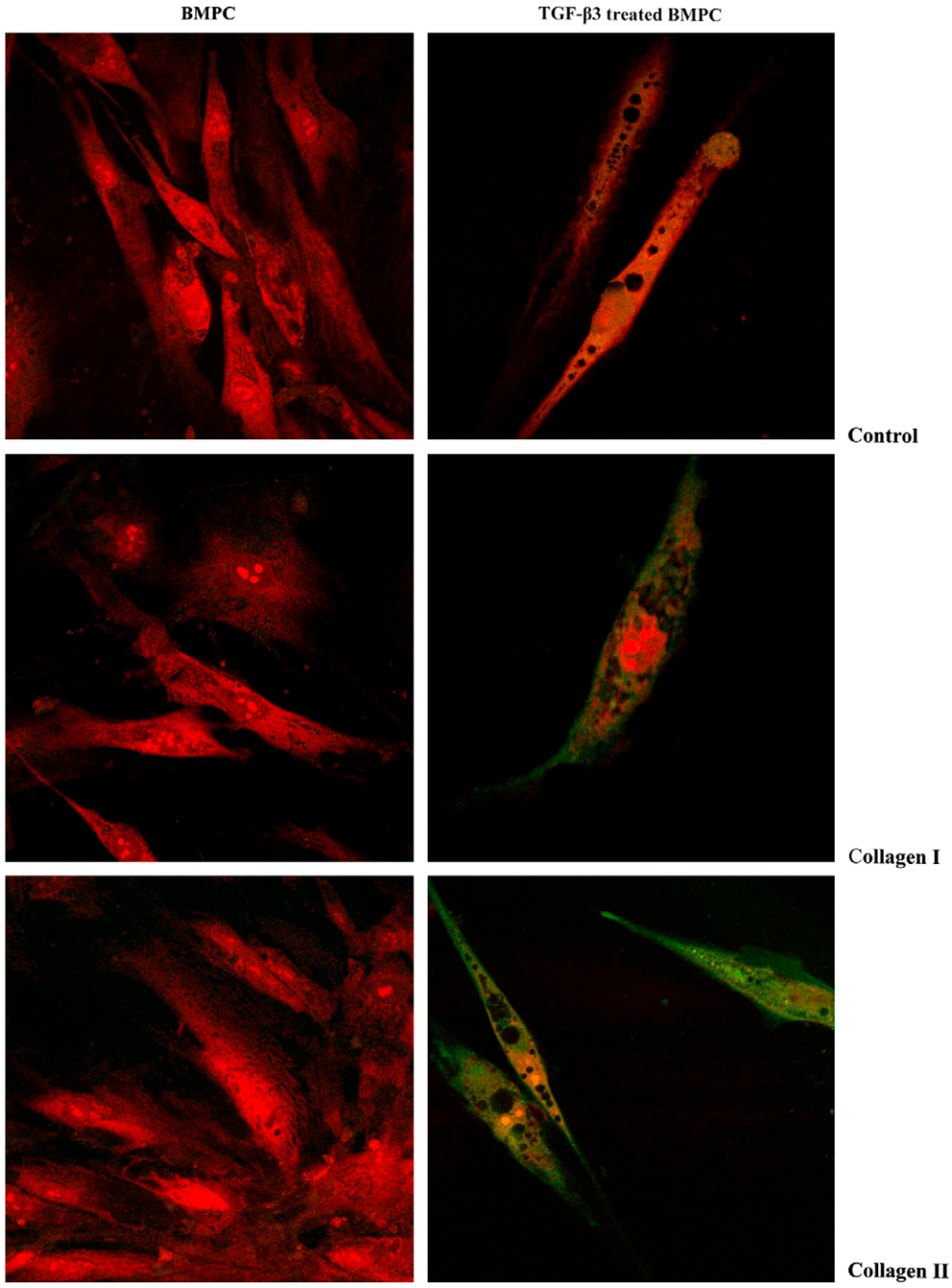

Figure 4 Immunofluorescence staining showing DNA presence in the cytoplasm and nucleus (red) and cytoplasmic localisation of collagen I and II precursors in TGF-β3 treated BMPC (green). (Magnification ×20).

It should also be noted that this study uses blood from relatively young volunteers. Age has not been shown to affect stem cell multipotency.[36,37] Further work, however, is required to determine if the number of these cells circulating reduces with donor age.

It is difficult to determine whether BMPCs are a synergistic population of different progenitors with a finite replication capacity or true multipotent stem cells. The former theory is supported by the wide variations in cell surface characteristics of cell populations isolated from blood.

This study demonstrates interesting findings regarding the chondrogenic potential of a unique $CD105^{+}CD14^{+}$ cell population isolated from the non-mobilised peripheral blood. This source of MPC shows a good potential for being used in tissue engineering cartilage. Demonstration of differentiation into several mesenchymal lineages is currently being investigated.

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Conflict of interest statement

We have no conflict of interest.

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