Isolating paediatric mesenchymal stem cells with enhanced expansion and differentiation capabilities

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ABSTRACT

Mesenchymal stem cells/marrow stromal cells (MSCs) are attractive for applications ranging from research and development to use in clinical therapeutics. However, the most commonly studied MSCs, adult bone marrow MSCs (A-MSCs), are limited by significant donor variation resulting in inconsistent expansion rates and multilineage differentiation capabilities. We have recently obtained permission to isolate paediatric MSCs (P-MSCs) from surplus iliac crest bone chips. Here, we developed a simple and easily replicable isolation protocol yielding P-MSCs which adhere to MSC defining guidelines. After confirming immunophenotypic marker expression we compared expansion rates, senescence, morphology and trilineage differentiation of P-MSCs to A-MSCs for multiple donors. We found P-MSCs have faster *in vitro* replication, consistently show significantly lower senescence and are capable of more reproducible multilineage differentiation than A-MSCs. We therefore believe P-MSCs are a promising candidate for use in research applications and potentially as part of an allogeneic therapeutic treatment.

INTRODUCTION

Over the last decades interest in mesenchymal stem cells/marrow stromal cells (MSCs) has grown; many have recognised their potential to advance scientific discovery and improve clinical treatment options [1-3]. MSCs unlike other lineage-committed progenitors or terminally differentiated cells are capable of multi-lineage differentiation which is desirable for a number of applications ranging from developmental research to use in advanced therapeutic medicinal products (ATMPs) [4-7]. MSCs are attractive for these applications as they can be easily isolated, cultured and expanded *in vitro* [2,8]. They have been found in a variety of tissues, blood and even urine [9-11]. Regardless of their point of isolation, MSCs must adhere to criteria determined by the International Society for Cellular Therapy (ISCT) as outlined by M. Dominici *et al.* [12]. Briefly, cells must (i) be plastic adherent, (ii) retain their multipotent differentiation capacity and (iii) express a panel of surface antigens. Although a diverse variety of MSCs meet these criteria, there are still numerous differences between populations depending on the method and tissue they are isolated from including variability in *in vitro* expansion, differentiation capability and cell surface marker expression [8,13-16]. These differences and the inherent donor variation observed between MSCs makes clinical translation and their use in ATMPs challenging.

In order for MSCs to be used as part of AMTPs, a sufficient quantity of cells must be obtained which are capable of producing consistent outcomes that satisfy the regulatory requirements set by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) [17-19]. When removed from the environment of their *in vivo* niche and expanded *in vitro*, MSCs rapidly lose their ability to replicate and differentiate, meaning their characteristics change unpredictably over time in culture [16,20-22]. This variation is often observed in bone marrow (BM) MSC populations which as of now is still considered the gold standard when it comes to MSCs [23,24]. Other MSC sources such as umbilical cord and adipose tissue are being actively characterised with promising but conflicting results [11,25-27]. An ideal MSC source would allow isolation with minimal patient discomfort and yield cells capable of reproducibly meeting EMA/FDA regulatory requirements [28]. Although BM MSCs are currently the gold standard for MSCs their isolation is associated with a painful procedure and harvesting of such material results in substantial patient discomfort and recovery time when used [27,29]. Many researchers, us among them, use surplus clinical material obtained from patients undergoing surgical procedures (total hip or knee replacement for example). However the age and disease status of the donors often negatively influences MSC performance [20]. Kretlow *et al.* found cell attachment, proliferation and differentiation were all affected as donor age increased [30,31]. MSCs from aged donors were less capable of secreting

and maintaining a chondrogenic matrix [32], and had a decreased bone forming potential *in vivo* [33]. It has also been reported cells from the elderly often exhibit cellular dysregulation which negatively impacts stem cell populations [31,34,35]. Additionally MSCs from elderly patients have been shown to have age-induced gene expression changes and earlier replicative senescence which further negatively effects MSC performance [31,36]. Cellular dysregulation in aging populations has also been hypothesised to add to the pathogenesis of these diseases which results in a damaged stem cell population [31,34]. As neither cell dysregulation or senescence is useful for research or the clinics, MSCs isolated from adult or geriatric populations are not an ideal cell source. MSCs isolated from younger patients have shown promise [11].

Recently we have gained access to small quantities of surplus bone from paediatric patients undergoing craniofacial reconstruction surgery from which we can easily isolate paediatric MSCs (P-MSCs). The resulting MSCs are plastic adherent, maintain MSC related immunophenotype and are capable of consistent differentiation. Here we outline how these cells are obtained, isolated and cultured as well as describe the morphological and phenotypic characteristics of these novel MSCs to allow others in the scientific community to utilise them for their own applications. We compare P-MSCs to adult MSCs (A-MSCs) isolated from BM and find P-MSCs to be capable of more consistent multilineage differentiation. We believe P-MSCs to be a promising candidate for use in both research as well as clinical applications.

MATERIALS AND METHODS

Mesenchymal stem cell isolation and expansion

All samples were harvested with the approval of the medical ethics committee at Erasmus Medical Centre (ErasmusMC, Netherlands). Adult-MSCs were isolated and expanded as previously described (MEC-2004-142 & MEC-2015-644) [13]. Paediatric mesenchymal stem cells (P-MSC) were isolated from leftover iliac crest bone chip material obtained from patients undergoing cleft palate reconstructive surgery (MEC-2014-16; 9-13 years). P-MSCs were isolated by gently swirling 10mL of expansion medium (αMEM containing 10 % serum (lot # 41Q204K, Gibco), 50 μg/mL gentamycin, 1.5 μg/mL fungizone, 25 μg/mL L-ascorbic acid 2-phosphate and 1 ng/mL fibroblast growth factor-2 (Instruchemie)) with iliac crest bone chips. Medium was removed and the process was repeated with an additional 10 mL expansion medium. The cell suspension from the combined medium of both washes was plated in a T75 flask and iliac crest chips were processed for histology. Flasks were washed 24 hours after plating with PBS to remove non- adherent cells and debris. Cells were cultured at 37°C and 5% carbon dioxide (CO_2). Expansion medium was

refreshed twice a week. P-MSCs were passed at approximately 80-90% confluency using 0.05% trypsin and replated at approximately 2,300 cells/cm^2. After passages 2-4 A-MSCs were used for trilineage differentiation and after passage 5 for FACs analysis, immunocharacterisation and β-galactosidase stainings. P-MSCs were always used after passage 5 unless otherwise noted (β-galactosidase staining, passage 8).

FACS analysis

A-MSCs and P-MSCs were trypsinised at passage 5 and rinsed in FACS flow. Cells were incubated for 30 minutes in 100 µL FACS buffer (BD Biosciences) containing antibodies against CD90 (APC), CD105 (FITC), CD73 (PE), CD271 (APC), CD166 (PE), HLA-DR (PerCP), HLA-ABC (FITC) or CD45 (PerCP). MSCs were washed with FACS flow, centrifuged at 689 g for 5 minutes, resuspended in 200 µL of FACS flow and analysed on a FACS Jazz flow cytometer (all antibodies BD Biosciences). Post-analysis was completed using FlowJo software version 10.0.7 (Treestar Inc.).

Senescence staining and quantification

The percentage of senescent cells was determined by staining for senescence-associated lysosomal β-galactosidase using a modification of Debacq-Chainiaux *et al*'s protocol [37]. A-MSCs and P-MSCs were seeded at 2,300 cells/cm^2 in complete expansion medium (as described above). On day 3 cells were refreshed and after 6 days cells were fixed in 1% [v/v] formaldehyde (Sigma) and 0.5% glutaraldehyde [v/v] (Sigma) in PBS for 15 minutes at 4°C. After washing with distilled water, cells were incubated for 24 hours at 37°C with 250 µL/cm^2 staining solution (1 mg X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Roche Diagnostics), 1.64 mg potassium hexacyanoferrate (III) (Sigma), 2.1 mg potassium hexacyanoferrate (II) trihydrate (Sigma), 2 µmol magnesium chloride hexahydrate (Sigma), 150 µmol sodium chloride, 7.3 µmol monohydrous citric acid (Sigma), 25.3 µmol disbasic dodium phosphate dihydrate (Sigma)) per mL distilled water; pH 6.0). After rinsing in distilled water, cells were counterstained with 1 g/L neutral red (Sigma) in a solution of 0.2% acetic acid. The number of positive cells was quantified and plotted relative to total cell number.

Chondrogenic differentiation

2x10^5 A-MSCs or P-MSCs were suspended in 500 µL of chondrogenic medium (high-glucose DMEM supplemented with 50 µg/mL gentamycin (Invitrogen), 1.5 µg/mL fungizone (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 40 µg/mL proline (Sigma), 1:100 v/v insulin-transferrin-selenium (ITS+; BD Biosciences), 10 ng/mL transforming growth factor β3 (Peprotech), 25 µg/mL L-ascorbic acid 2-phosphate (Sigma), and 100 nM dexamethasone (Sigma)) in 15 mL polypropylene tubes.

Samples were centrifuged at 200 g for 8 minutes. Medium was replaced twice weekly for 21 days (P-MSCs) or for 28-35 days (A-MSCs). Samples were formalin fixed for histology (4% (w/v) formaldehyde in PBS for 2 hours).

Osteogenic differentiation

A-MSCs or P-MSCs were plated at a density of $3x10^3$ cells/cm^2 in expansion medium (previously described). 24 hours following seeding medium was replaced with osteogenic induction medium (high-glucose DMEM supplemented with 10% serum, 50 µg/mL gentamycin, 1.5 µg/mL fungizone, 10 mM glycerol phosphate (Sigma), 0.1 µM dexamethasone (Sigma), and 0.1 mM L-ascorbic acid 2-phosphate (Sigma)). Medium was refreshed as previously described for 14-21 days, depending on when cell sheets began to pull away from the outer perimeter of the well or when calcium deposition was observed macroscopically, at which point the culture was ended. Cells were cultured at 37°C and 5% CO_2. Samples were prepared for histology (fixed in 70% EtOH at 4ºC) following the end of culture.

Adipogenic differentiation

A-MSCs or P-MSCs were plated at a density of $2.1x10^4$ cells/cm^2 in adipogenic induction medium (high-glucose DMEM supplemented with 10 % serum, 50 µg/mL gentamycin, 1.5 µg/mL fungizone, 0.2 mM indomethacin (Sigma), 0.01 mg/mL insulin (Sigma), 0.5 mM 3 iso-butyl-1-methyl-xanthine (Sigma)). Medium was refreshed as previously described and cells were maintained at 37°C and 5% CO_2. Samples were prepared for histology (fixed in 4% (w/v) formalin) following harvest.

Haematoxylin-eosin staining

Paediatric bone chips were fixed for 24 hours in 4% (w/v) formalin, decalcified in 10% EDTA (w/v) for 30 days and paraffin embedded. Chondrogenic MSC pellets were fixed in 4% (w/v) formalin for 2 hours and paraffin embedded. 6 µm-thick sections were cut, deparaffinised and stained with haematoxylin-eosin (H&E). H&E staining was performed by incubating deparrafinised samples in Gill's haematoxylin (Sigma) for 5 minutes, washed in none distilled water for 5 minutes, washed in distilled water, and counterstained for 45 seconds with 2% Eosin (Merck; in 50% ethanol, 0.5% acetic acid). Samples were fixed in 70% EtOH for 10 seconds and rehydrated (sequentially in 96% EtOH, 100% EtOH, and xylene for 1 minute). Samples were mounted in Entellan (Depex).

Thionine staining

Deparaffinised samples were incubated in 0.04% thionine (prepared in 0.01M sodium acetate, pH 4.5) for 5 minutes, differentiated in 70% EtOH for 10 seconds

and then rehydrated as previously described. Samples were mounted in Entellan (Depex).

von Kossa staining

Osteogenically differentiated MSCs were fixed in 4% (w/v) formalin for 1 hour. Following a rinse with ultrapure water, samples were incubated in 5% w/v silver nitrate (Sigma) for approximately 30 minutes under direct light provided by a light box. Following incubation samples were washed in ultrapure water and counter-stained with nuclear fast red (Merck) for five minutes. Samples were dehydrated in 70% EtOH for 10 seconds followed by 96% EtOH and 100% EtOH for one minute. Samples were imaged in 100% EtOH directly following staining.

Oil red staining

Following a 15 minute fixation in 4% (w/v) formalin samples were rinsed in distilled water for 10 minutes. 0.5% w/v Oil-red O (in 2-propanol; Sigma) was added to samples for 10 minutes followed by further rinsing with distilled water. Samples were imaged in distilled water.

P-MSC-PBMCs co-culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy male donors (Sanquin, Rotterdam) by a Ficoll-Paque PLUS gradient separation as previously described [38] (GE Healthcare). PBMCs were resuspended in human serum conditioned medium (HCM) (RPMI-1640 medium, 1% GlutaMAX (Life Technologies), 50 µg/mL gentamycin, 1.5 µg/mL fungizone, 10% human serum (Sigma-Aldrich)) and stored at -80ºC until use. P-MSCs were trypsinised as previously described and seeded in low-evaporation round bottom 96 well plates. 24 hours following seeding, $1x10^7$ PBMCs were labelled with 20 µL of CFSE. T cells were stimulated by adding anti-CD3/CD28 antibodies (1 µL/10^6 cells) to PBMC suspension with an anti- goat linker antibody (2 µl/10^6 cells). Stimulated 100,000 PBMCs were co-cultured with P-MSCs at a P-MSC:PBMC ratios of 1:2.5, 1:5, 1:10 or 1:20., PBMCs were harvested 5 days later and stained with CD3-PerCP (clone SK7), CD8- PE-Cy7 and CD4-APC (BD Biosciences). Samples were fixed in 3.6% paraformaldehyde and analysed using a FACS Jazz flow cytometer (BD Biosciences) and post-analysis was completed using FlowJo software version 10.0.7 (Treestar Inc.). Data is represented as reciprocal of the mean fluorescence intensity (MFI).

Statistical analysis

Mann Whitney U analysis was performed using SPSS (Ver 21. SPSS Inc, Chicago, USA) on data used in figures 1, 2, and 3. Kruskal-Wallis analysis with Dunn's mul-

tiple comparison was performed on figure 4. Data are shown as mean ± SD, P-values under 0.05 were considered significant.

RESULTS

Paediatric MSCs express a panel of established MSC cell surface markers

P-MSCs were isolated from small quantities of surplus bone biopsies from patients undergoing cleft pallet reconstruction surgery. The environment from which the P-MSCs are isolated contains both bone and bone marrow elements (figure 1).

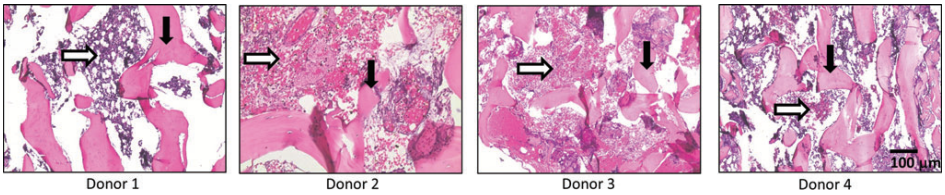

Figure 1. P-MSCs are isolated from illiac crest rest material containing both bone and bone marrow.

Hematoxylin and Eosin stained sections of the illiac crest chips from which P-MSCs are isolated. The cell source environment is rich in both bone marrow elements and bone (black arrows indicate bone, white arrow bone marrow).

In order to prove isolated cells were indeed true MSCs we characterised the immunophenotypic expression of common MSC markers. These markers included a panel which are known to be expressed on MSCs including CD105, CD90, CD73, CD271, CD166 and HLA-ABC as well as a commonly used negative marker, lymphocyte associated CD45 [12,39]. Both A-MSCs and P-MSCs were analysed following 5 passages. MSC markers were expressed at a similar level in P-MSCs and A-MSCs (figure 2).

Both populations were negative for CD45, and positive HLA-ABC. About half the P-MSCs and A-MSCs population was positive for HLA-DR which was not surprising as HLA-DR expression can increase with *in vitro* culture of MSCs [40]. We found no significant difference in HLA-DR expression between P-MSCs and A-MSCs (figure 2).

Paediatric MSCs have enhanced expansion properties compared to adult MSCs

During expansion P-MSCs exhibit a typical MSC morphology similar to that observed in A-MSCs (figure 3a). Although the total number of days A-MSCs and P-MSCs took to establish the initial culture (figure 3b) and reach passage 3 (figure

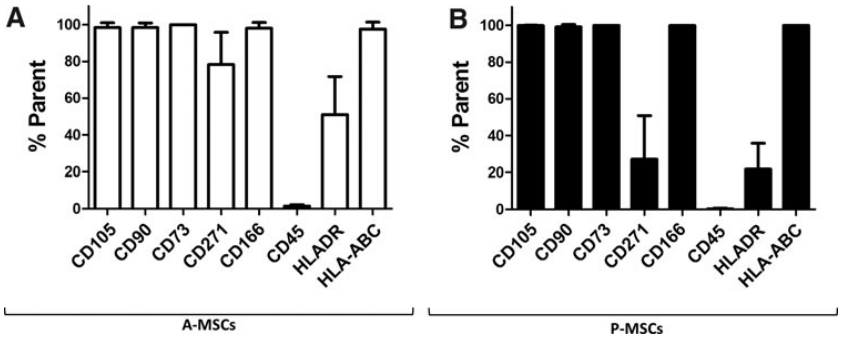

Figure 2. P-MSCs and A-MSCs express similar levels of general stem cell markers.

General MSC related markers which are commonly expressed on A-MSCs **(A)** are also expressed at a similar level in P-MSCs **(B)** (N.S. differences between A-MSCs and P-MSCs; Mann Whitney U test) Both populations are negative for haematopoietic marker CD45 however are positive for a panel of other immune related markers.

3c) did not change, P-MSCs expanded significantly faster than A-MSCs, yielding more cells after the same time in culture (figure 3d; $p<0.02$). This difference in cell number could be attributed to cell size. A-MSCs enlarged the longer they were in culture, whereas P-MSCs remained small (figure 3a, 4a).

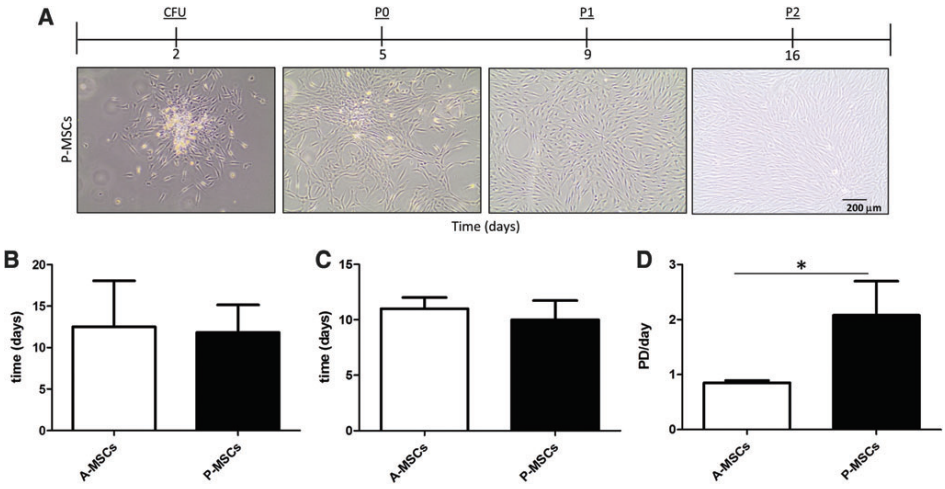

Figure 3. P-MSCs and A-MSCs have a similar rate of expansion but P-MSCs undergo more population doublings.

A) Cell morphology typically observed during expansion of P-MSCs (representative donor). **B)** Total days taken to establish culture from plating initial cell suspension to passage 0 does not differ between P-MSCs and A-MSCs. **C)** Expansion time from passage 0 to passage 3 does not differ between A-MSCs and P-MSCs. **D)** Total number of population doublings between passage 1 and passage 3 is greater in P-MSCs compared to A-MSCs ($p<0.0238$; Mann Whitney U test). (Abbreviations: CFU-colony forming units;P0: passage 0;P1: passage 1; P2: passage 2; P3: passage 3; PD: population doublings)

Paediatric MSCs are a less senescent cell source compared to A-MSCs

As increased cell size is a hallmark of senescence, a permanent cell cycle arrest that A-MSCs have been shown to undergo *in vitro*, we compared senescence between A-MSCs and P-MSCs [41,42] We observed cell enlargement of A-MSCs compared to P-MSCs (figure 4a). Senescence-associated lysosomal β-galactosidase staining

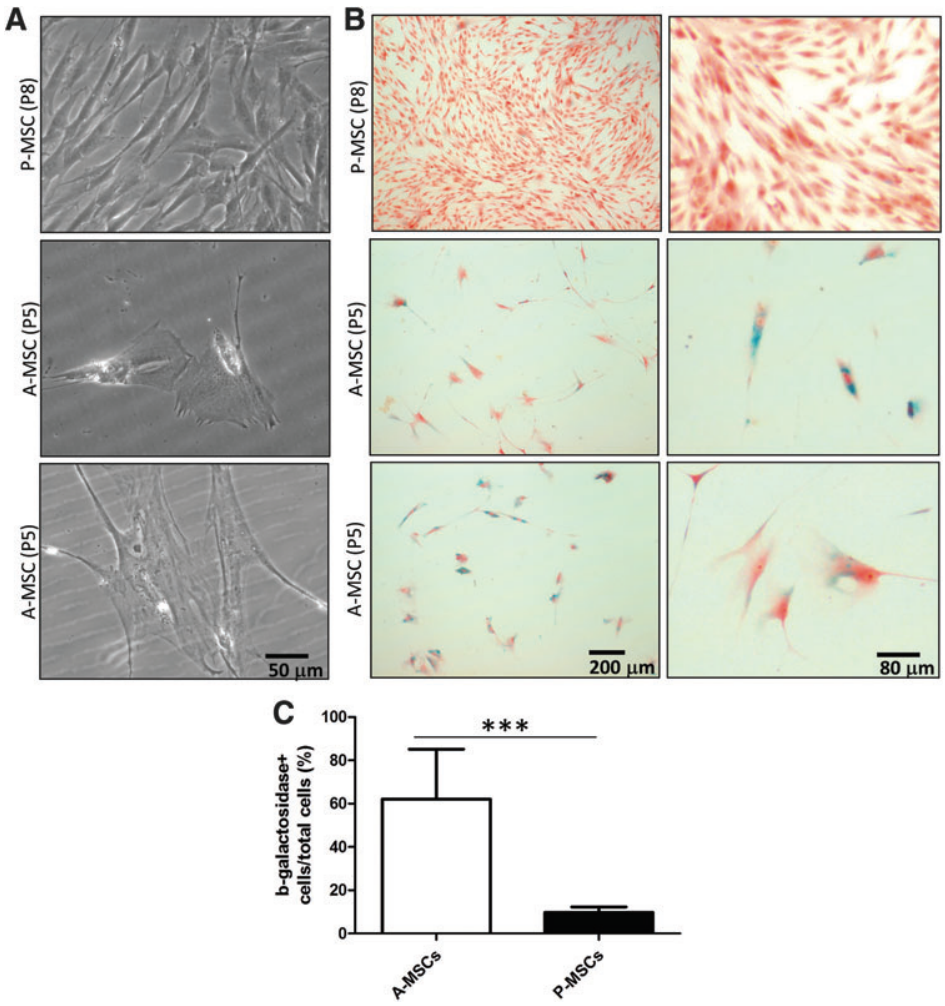

Figure 4. P-MSCs contain significantly less senescent cells than A-MSCs.

A) Cell morphology observed during expansion of P-MSCs (p8) and of A-MSCs (p5). A-MSCs display a larger cell morphology compared to P-MSCs even though they are an earlier passage. Both A-MSCs and P-MSCs were seeded at the 2,300 cells/cm^2 and expanded under normal conditions for 6 days. **B)** b-galactosidase staining of both P-MSCs (p8) and A-MSCs (p5). P-MSCs contain far less positively b-galactosidase stained cells than A-MSCs. **C)** Percentage of senescent cells counted in P-MSCs is significantly lower than that of A-MSCs (n=4; p<0.000; Mann Whitney test). (Abbreviations: P5: passage 5, P8: passage 8)

showed P-MSCs, even after extensive passage (p8; figure 4b,c), contained significantly less senescent cells than A-MSCs at an earlier passage (p5; figure 4b,c).

In monolayer P-MSCs reduce T cell proliferation at a similar level as A-MSCs

Our lab has previously shown that A-MSCs repress the proliferation of allogeneic T cells, a feature essential for many anti-inflammatory MSC-based therapeutics [38]. To examine if P-MSCs repress T cell proliferation, allogeneic T cells from PBMC fractions were CD3/CD28 stimulated and added in suspension to P-MSC monolayers.. In both CD4+ and CD8+ T cell subsets, P-MSCs inhibited T cell proliferation in a dose-dependent manner and to similar extend as we have previously reported for A-MSCs (figure 5). Here we found P-MSCs exhibited a similar level of inhibition to what was reported by A-MSCs [38].

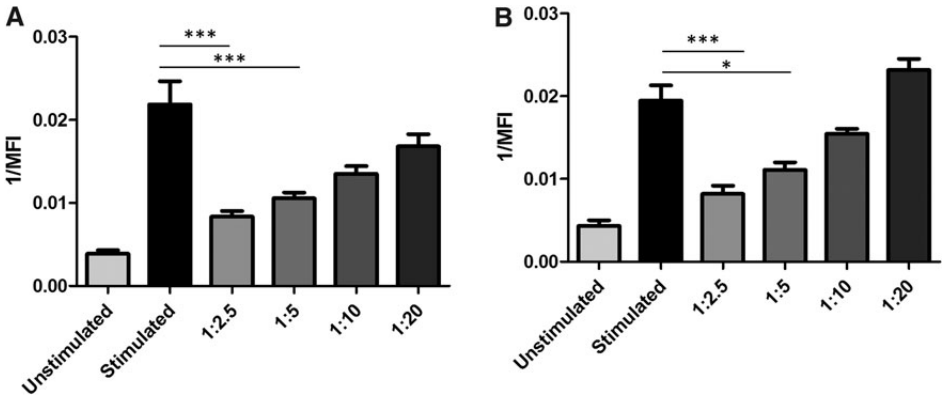

Figure 5. P-MSCs reduce allogeneic CD4+ and CD8+ T cell proliferation in monolayer.

Stimulated (+CD3/CD28) PBMCs co-cultured with P-MSCs at different MSC:PBMC ratios (1:2.5, 1:5, 1:10, 1:20). CD4+ **(A)** and CD8+ **(B)** proliferation rates were found to decrease in a dose dependent manner following 5 days in culture. (N=3 P-MSC donors N=2 PBMC donors) Abbreviation: MFI-mean fluorescent intensity. (Kruskal-Wallis with Dunn's post hoc correction; ***P>0.001, *P>0.05)

Paediatric MSCs exhibit more consistent multilineage differentiation capacity compared to adult donors

A-MSCs are known to exhibit inconsistent differentiation capabilities which varies greatly between donors. This severely limits their use in applications where consistency is essential [43]. Compared to A-MSCs donors (passages 2-5; figure 6a) the trilineage differentiation potential of P-MSCs (passage 5; figure 6b) was found to be more consistent. Even though P-MSCs were used several passages beyond that used for the A-MSCs, P-MSCs more consistently underwent adipogenesis, osteogenesis and chondrogenesis with only 1 out of 12 donors not being able to make bone or cartilage following treatment (figure 6b). A-MSCs showed much more variability in

their differentiation potential. Out of the 14 A-MSC donors tested, 3 donors failed to undergo adipogenesis, 5 donors failed to osteogenically differentiate and 5 donors were unable to chondrogenically differentiate (figure 6a).

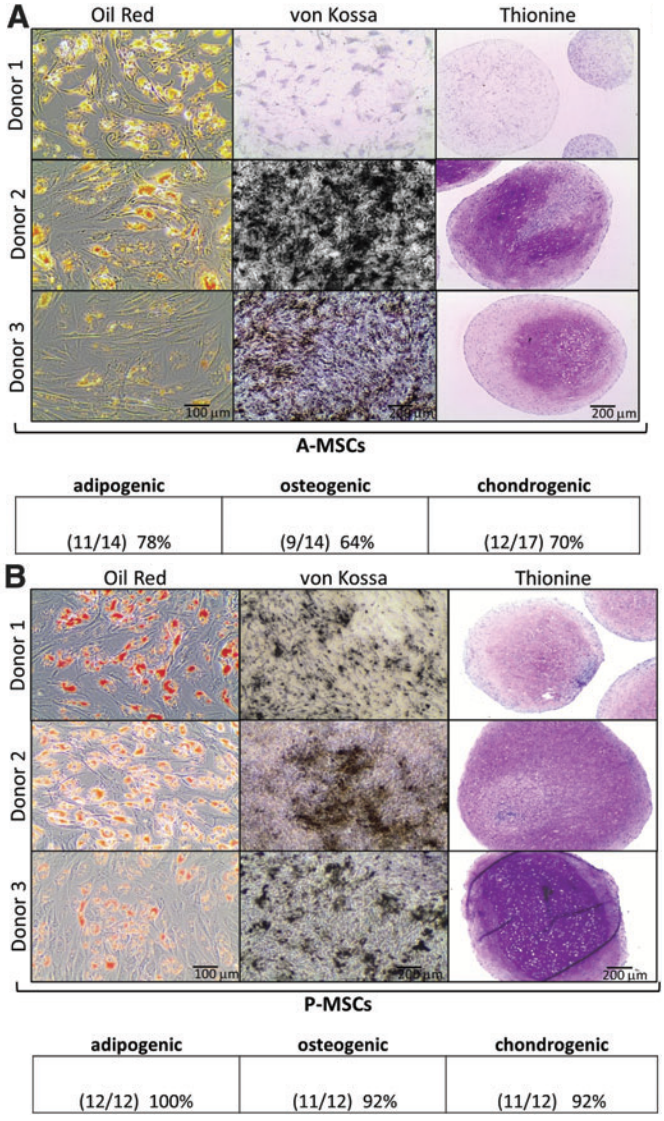

Figure 6. P-MSCs show more consistent capacity for multilineage differentiation.

A) A-MSCs (passage 2-4) show great variation in differentiation capacity. A-MSCs are known to exhibit far greater variation in differentiation capacity (Chamberlain, Fox et al. 2007; Noort, Scherjon et al. 2003). **B)** P-MSCs (passage 5-6) are capable of tri-lineage differentiation with all donors but one being capable of osteogenic and chondrogenic differentiation. All P-MSCs tested could adipogenically differentiation.

DISCUSSION

Identifying cell sources with enhanced and reliable differentiation capabilities and expansion properties is necessary in order for MSCs to be more readily utilised both in research and in ATMPs. Here we have established a simple isolation protocol to obtain P-MSCs from surplus iliac crest bone chip material (figure 1). We confirmed P-MSCs expressed cell surface markers typically used to properly identify MSC populations. P-MSCs showed similar expression levels of general stem cell markers CD105, CD90, CD73 and CD166 compared to A-MSCs, as well as the absence of the hematopoietic marker CD45 (figure 2). As these markers are conventionally used to identify MSCs [44,45] we are confident that this marker expression in combination with other results presented here we have proved P-MSCs are indeed MSCs. In this study A-MSCs displayed a higher expression of CD271 and HLA-DR compared to P-MSCs. It has been previously reported that in MSCs CD271 as well as HLA-DR expression decrease over time in culture [46,47]. As P-MSCs undergo more population doublings compared to A-MSCs at the same passage (figure 3d) this increased cellular division could have contributed to the decreased expression of both CD271 and HLA-DR we observe in P-MSCs.

P-MSCs expanded more rapidly than A-MSC donors, which might be attributed to the relatively low senescence in P-MSCs (figure 4). Senescent cells are much larger than non-senescent cells [48]. A-MSCs have more enlarged senescent cells which do not divide [49,50] making it easy to understand why the population doublings in A-MSCs are effected. Additionally, by preventing proliferation, senescence can also blocks differentiation pathways requiring proliferation, such as chondrogenic differentiation [51]. Being able to obtain cells with higher proliferation and differentiation capacity in a shorter time than is possible with A-MSCs makes P-MSCs an attractive cell source.

If P-MSCs are to be utilised in an allogeneic setting it is important to show P-MSCs maintain immunomodulatory capabilities typically observed in A-MSCs [38,52]. MSCs are known to be able to manipulate T-cell proliferation and phenotypic behaviour and their immunosuppressive nature makes them an interesting candidate from a clinical perspective [52-54]. In this study we found P-MSCs were capable of inhibiting T-cell proliferation at a similar level to what we previously reported with A-MSCs using a 1:5 ratio (MSC:PBMCs) [38]. For use in an allogenic model it is advantageous for P-MSCs to inhibit T-cell proliferation to prevent an unwanted immune reaction following transplantation. However how P-MSCs interact with other immune cell types including antigen presenting cells needs to be determined in order to further understand how they would respond to a fully functional immune system.

A-MSCs have been reported to have inconsistent multilineage differentiation capabilities [15,20,55]. P-MSCs were capable of more consistent multilineage differentiation compared to the A-MSCs in this study. Senescence could have contributed in part to the difference we observed here as senescent MSC cell populations undergo phenotypic changes [56,57] and exhibit chromosomal abnormalities [58,59] which ultimately could influence their differentiation capacity [51]. In this study it is plausible that a combination of factors influenced the differentiation capacity of these cell populations. It is logical cells from a younger, healthier patient which contain less senescent cells would be capable of better multilineage differentiation than senescent cells obtained from elderly patients. Having a cell source with more consistent differentiation capacities is ideal as it allows for more reproducible results.

Here we have described an easy isolation protocol which allows access to a P-MSC population with enhanced expansive and differentiation potential compared to A-MSCs. P-MSCs with their rapid expansion, low senescence and consistent multilineage differentiation are therefore prime candidates for applications from drug screening and development to use in ATMPs.

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DISCLOSURE STATEMENT

Authors declare there is no conflict of interest.

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