The assessment of CD146-based cell sorting and telomere length analysis for establishing the identity of mesenchymal stem cells in human umbilical cord [version 2; peer review: 2 approved]

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Abstract

Adult stem cells are characterised by longer telomeres compared to mature cells from the same tissue. In this study, candidate CD146⁺ umbilical cord (UC) mesenchymal stem cells (MSCs) were purified by cell sorting from UC tissue digests and their telomere lengths were measured in comparison to donor-matched CD146-negative fraction.

UC tissue fragments were enzymatically treated with collagenase and the cells were used for cell sorting, colony-forming fibroblast (CFU-F) assay or for long-term MSC cultivation. Telomere lengths were measured by qPCR in both culture-expanded MSCs and candidate native UC MSCs. Immunohistochemistry was undertaken to study the topography of CD146⁺ cells.

Culture-expanded UC MSCs had a stable expression of CD73, CD90 and CD105, whereas CD146 declined in later passages which correlated with the shortening of telomeres in the same cultures. In five out of seven donors, telomeres in candidate native UC MSCs (CD45⁻CD235α⁻CD3¹⁻CD146⁺) were longer compared to donor-matched CD146⁻ population (CD45⁻CD235α⁻CD3¹⁻CD146⁻). The frequency of CD45⁻CD235α⁻CD3¹⁻CD146⁺ cells measured by flow cytometry was ~1000-fold above that of CFU-Fs (means 10.4% and 0.01%, respectively). CD146⁺ cells were also abundant in situ having a broad topography including high levels of positivity in muscle areas in addition to vessels.

Although qPCR-based telomere length analysis in sorted populations...
could be limited in its sensitivity, very high frequency of CD146⁺ cells in UC tissue suggests that CD146 expression alone is unlikely to be sufficient to identify and purify native MSCs from the UC tissue.
We are very grateful to the reviewers’ for their careful reading of our work and for their insightful comments.

We can confirm that we did perform FSC/SSC gating to remove anuclear red cells and cellular debris in all our sorting experiments. We have now modified the relevant section of our Materials and Methods as well as Figure 2A to reflect this (gate R1). Additionally, we have added average percentage of cells within R1 out of all events collected into the appropriate Figure Legend.

In our experiments with UC tissue digests, a distinct CD45 CD235a-CD31 CD146+ population was always evident above the isotype control staining. We have made new flow cytometry plots for panel 2A to reflect all of these changes (FSC/SSC gating and isotype control panel).

We have now provided information on CD146+ and CD146–fractions’ cell yields in Figure 2B, left panel and we confirm that we have performed ten sorting experiments. Fraction purities post-sort are always >95% using our cell sorter (see Churchman et al., Arthritis and Rheumatism, 2012, 64:2632).

Our method was based upon that of Cawthon 2002, however when using unexpanded cells sorted from UC the cell numbers were limited because of the amount of tissue processed and inevitable cell losses caused by multiple centrifugation steps prior to cell sorting. Due to this we elected to standardise our telomere length experiments to 1000 sorted cells per fraction (previously overlooked from methods). We now present normalized T/S ratios adjusted according to Cawthon 2002 paper in Figure 2C and conversion to Cawthon 2002 units. We have also strengthened this figure with information on methods. We now present normalized T/S ratios adjusted according to Cawthon 2002 paper in Figure 2C and conversion to Cawthon 2002 units.

We now recognize the potential of CD146 marker in the UC tissue and to purify candidate MSCs from UC tissue digests based on the non-haematopoietic (CD45 CD235α–), non-EC (CD31–), CD146+ phenotype. We measured telomere lengths in CD146– and donor-matched control CD146+ populations using quantitative real-time PCR (qPCR) and examined the topology of CD146+ cells in situ using immunohistochemistry.

**Materials and methods**

**Patients and cells**

UC tissue was collected from the UCs of consenting full-term caesarean section patients (n=10). After delivery, UCs were immediately stored in Dulbecco’s phosphate buffered saline (DPBS, #14190-250, Invitrogen, Renfrew, UK) at 4°C. All samples were obtained after written informed consent and the protocols were approved by National Research Ethics Committee (07/Q1205/27).

Human primary skin fibroblasts were obtained from Lonza and ATCC (Lonza, Cambridge, UK and ATCC, Middlesex, UK).

**MSC and EC isolation from UC tissue**

The whole UC tissue was mechanically dissected in small pieces (~0.24±0.1 g) and washed repeatedly with DPBS followed by enzymatic digestion using 600 U/ml collagenase I (#07902, Stem Cell Technologies, Grenoble, France) for 6 hours. Released cells were resuspended in 1:50 v/v DPBS, filtered through a 70 μm cell strainer (#352350, BD Biosciences, Oxford, UK), centrifuged (650 g) and counted.

For primary MSC culture, UC tissue digestes were seeded in 6-well tissue culture plates (#3516, Corning, Amsterdam, The Netherlands) in non-haematopoietic (NH) expansion medium (#130-091-680, Miltenyi Biotec, Bisley, UK) at a density of 5x10⁶ cells/well. After observing 80% cell confluency [denoted passage (p) 0], adherent cells were trypsinised using 0.5% trypsin/EDTA (#15400-054, Invitrogen) and re-seeded at 5x10⁵ cells/cm² flask for further passaging which was performed as 1:1 splits until approximately p17. Cultures were fed twice weekly with half media changes. The number and viability of the cells at each passage were evaluated using Trypan blue staining. For primary EC culture, 5x10⁶ cells were seeded into 6-well tissue culture plates coated with 2 μg/cm² fibronectin (#354008, BD Biosciences) in endothelial basal medium (EBM2, #CC-3162, Lonza). On day 2, adherent cells were washed with DPBS and subsequently fed three times per week with half media changes. ECs were passages using 1:1 splits until p4.

**Introduction**

Perinatal mesenchymal stem cell (MSC) sources are attracting increasing attention as an alternative to ‘gold standard’ bone marrow (BM) MSCs. Owing to its universal accessibility, umbilical cord (UC) tissue represents an attractive source of MSCs for cell therapy¹. However, the potency of standard culture-expanded UC MSCs, especially towards osteoblasts, chondrocytes and adipocytes, is lower compared to BM MSCs²,³. This can be explained by the fact that conventional UC MSC cultures arise from diverse clonal populations that possess varying degrees of self-renewal leading to mixed cultures that gradually lose their MSC properties⁴. The therapeutic advantages of minimally expanded UC MSCs would therefore include a better preservation of their native functionality as well as rapid manufacture and reduced cost⁵. However, no agreement yet exists on the native phenotype of UC MSCs⁶–⁷, which is an essential pre-requisite for their isolation and minimal expansion.

Prospective isolation of UC MSCs has been attempted after enzymatic digestion followed by selection for specific cell surface markers using fluorescence-activated cell sorting (FACS) or immunomagnetic bead separation techniques. Previous studies showed that CD45-based negative depletion¹, neural ganglioside (GD2)− and CD146-based positive selection⁸,⁹ could be used to select for clonogenic and multipotential MSC fractions. Although CD146 is expressed on pericytes, which have been proposed as a reservoir of tissue specific progenitors and MSCs¹⁰,¹¹, it is also present on the surface of CD31+ UC endothelial cells (ECs)¹²,¹³, and therefore cannot be used alone to achieve high-purity of UC MSC preparations.

Whilst the majority of studies in this field used combinations of surface markers to isolate putative MSCs from different tissues, followed by their long-term clonal expansion and analysis of multipotentiality¹⁴, another alternative and faster approach could involve measuring the telomere length of freshly-sorted candidate MSC fractions. As immature cells, MSCs by definition should have longer telomeres compared to mature cell types. This idea was first proposed by Flores et al. (2008) who showed that stem cell compartments from hair follicles, intestine, testis, cornea and brain are enriched with cells with the longest telomeres¹⁵.

The aims of this study were to confirm that CD146 could be a good MSC marker in the UC tissue and to purify candidate MSCs from UC tissue digests based on the non-haematopoietic (CD45 CD235α–), non-EC (CD31–), CD146+ phenotype. We measured telomere lengths in CD146– and donor-matched control CD146+ populations using quantitative real-time PCR (qPCR) and examined the topography of CD146+ cells in situ using immunohistochemistry.

For primary MSC culture, UC tissue digestes were seeded in 6-well tissue culture plates (#3516, Corning, Amsterdam, The Netherlands) in non-haematopoietic (NH) expansion medium (#130-091-680, Miltenyi Biotec, Bisley, UK) at a density of 5x10⁶ cells/well. After observing 80% cell confluence [denoted passage (p) 0], adherent cells were trypsinised using 0.5% trypsin/EDTA (#15400-054, Invitrogen) and re-seeded at 5x10⁵ cells/cm² flask for further passaging which was performed as 1:1 splits until approximately p17. Cultures were fed twice weekly with half media changes. The number and viability of the cells at each passage were evaluated using Trypan blue staining. For primary EC culture, 5x10⁶ cells were seeded into 6-well tissue culture plates coated with 2 μg/cm² fibronectin (#354008, BD Biosciences) in endothelial basal medium (EBM2, #CC-3162, Lonza). On day 2, adherent cells were washed with DPBS and subsequently fed three times per week with half media changes. ECs were passages using 1:1 splits until p4.

**See referee reports**
For all cultures, the number of population doublings (PDs) between passages (starting from initial passage: p0) was calculated according to the following equation: PD = log 2 (Nt/Ni), where Ni and Nt are the initial and terminal cell counts, respectively. PDs before p0 were calculated based on colony-forming unit-fibroblast (CFU-F) potential of cells seeded and the number of cells at p0 according to the equation PD = log 2 (N cells at p0/N seeded CFU-Fs). The CFU-F assay was performed in triplicate at the cell seeding density of 5x10^4 cells/well and individual colonies (>50 cells) were counted on day 14 after staining with 1% w/v Crystal Violet (#V5265, Sigma, Hertfordshire, UK).

**Gene expression analysis of UC MSCs**

RNA extraction from culture expanded UC MSCs (p4) and fibroblasts was performed using RNA/DNA/Protein purification kit (#23500, Norgen, Ontario, Canada) according to the manufacturer’s instructions and RNA yield was quantified by using NanoDrop 2000 spectrophotometer (Thermo, Essex, UK). Single strand cDNA was synthesised using high-capacity cDNA reverse transcription kit (#4368814, Applied Biosystems, Warrington, UK).

A custom designed 48 gene Taqman low density array (TLDA, Applied Biosystems) contained mesenchymal and endothelial lineage-related transcripts and novel surface receptors that could be used to segregate MSCs from fibroblasts based on previously published data. To perform TLDA, 200 ng cDNA were used per port. The results were obtained using an ABI PRISM 7900HT SDS (Applied Biosystems). Normalisation of transcript levels relative to reference gene HPRT was performed using the formula: 2^ΔΔCt, ΔCt= Ct value (selected transcript) - Ct value (HPRT).

**Immunophenotyping of MSC and EC cultures**

Phenotypic characterisation was performed on culture expanded MSCs at different passages and on cultured ECs at p4 using: CD31-FITC (#MCA1738F), CD105-PE (#MCA1557PE), CD90-PE (#MCA90PE) (all from Serotec, Kidlington, UK), CD73-PE (#550257), CD146-PE (#550315) (both from BD Pharmingen, Oxford, UK), and CD271-PE (#130-091-885, Miltenyi Biotec). The isotype controls were IgG1-FITC (#550616, BD Pharmingen) and IgG1-PE (#MCA928PE, Serotec). A total of 2x10^5 cells was stained with 5 μl FITC- or PE-conjugated antibodies, and dead cells were excluded using 2 μg/ml propidium iodide (PI, #P1304MP, Invitrogen). Cells were acquired using FACScan equipped with CellQuest software version 3.1 (BD Biosciences) and the proportions of the different fractions were calculated as a percentage of total live cells.

**Fluorescence-activated cell sorting of candidate UC MSCs**

Cell sorting was performed using a MoFlo cell sorter equipped with SUMMIT software (Beckman Coulter, Buckinghamshire, UK). Following collagenase digestion of UC tissue, 2x10^5 cells were split into two tubes. One tube was stained with 5 μl of neat CD45-FITC (#F0861), CD235α-FITC (#F0870) (both from DAKO, Cambridge, UK), CD146-PE (BD Pharmingen) and CD31-APC (#130-092-652, Miltenyi Biotec), whereas the other was stained with 2.5 μl of neat isotype controls IgG1-FITC (#550617, BD Pharmingen), IgG1-PE (#MCA928PE, Serotec) and IgG1-APC (#130-098-846, Miltenyi Biotec). After incubation with relevant antibodies and washes, 2 μg/ml 7-aminoactinomycin D (7-AAD) (#A1310, Invitrogen) was added to exclude dead cells before sorting into four fractions: haemopoietic cell fraction (HC), CD45^-CD235α^-CD31^- EC fraction, CD45^-CD235α^-CD31^-; candidate MSC fraction, CD45^-CD235α^-CD31^-CD146^- and non-MSC fraction, CD45^+CD235α^-CD31^-CD146-. The latter two subsets were processed for telomere length analysis.

**Telomere length measurements**

QIAamp DNA Mini kit (#51306, Qiagen, Crawley, UK) was used for gDNA extraction from cultured MSCs and freshly-sorted CD146^- and CD146+ subsets. Samples were run in triplicate using 20 ng gDNA for expanded cells and all gDNA extracted from 1000 cells for the freshly sorted CD146^- versus CD146+ subset comparison. Telomere length measurement by SYBR Green qPCR (#4309155, Invitrogen) involved determining the relative ratio of telomere repeat copy number (T) to a single copy gene (36B4) copy number (S): T/S ratio, as previously described.

**Immunohistochemistry**

Immunohistochemistry was used to characterise UC tissue architecture and investigate CD146 cell topography in situ to ascertain whether it exhibited the proposed MSC pericyte distribution. Whole UC tissue cross sections were embedded in OCT mounting media (#361603E, VWR, Leicestershire, UK), snap frozen in liquid nitrogen and stored at -80°C. Cryostat sections (6 μm) were mounted on superfrost slides (#48311-703, VWR) and dried overnight at 37°C. Immunohistochemistry was performed using DAKO REAL detection system (#K4065, DAKO) according to the manufacturer’s instructions. Primary antibodies included: CD31 (#CBL468, working concentration 1:10), CD146 (#MAB16985, working concentration 1:20) (both from Chemicon, Watford, UK), CD34 (#M716501, working concentration 1:100), CD271 (#M3507, working concentration 1:20) (both from DAKO). Antibody binding was visualized using DAKO REAL DAB+ chromogen (#K3468, DAKO) and slides were counterstained by mounting in Harris haematoxylin (#HHS128, Sigma). Slides were mounted using DPX (#317616, Sigma) and images were captured using CAMEOCA C-7070 camera (Olympus, Tokyo, Japan).

**Statistical analysis**

The software used for analysis and statistics was GraphPad (Graph-Pad software, La Jolla, USA). The gene expression results were analysed with Mann-Whitney test for unpaired samples (P<0.05: high significance, 95% confidence interval). Sorted fraction yields were compared using Wilcoxon matched pairs test. The cell sorting and CFU-F results were analysed by Kruskal-Wallis test with high significance, 95% confidence interval. Sorted fraction yields were compared using Wilcoxon matched pairs test. The cell sorting and CFU-F results were analysed by Kruskal-Wallis test with high significance, 95% confidence interval.

**Results**

**Molecular profile of culture-expanded UC MSCs compared to fibroblasts**

We initially aimed to confirm the validity of CD146 as a candidate marker of UC MSCs using cultures established following standard MSC protocols. Mesenchymal tri-potentiality of these cultures was demonstrated in our previous study. We studied the expression of...
Expression of CD146 and CD271 during extended culture of UC MSCs
Having shown NGFR and MCAM transcript expression in culture-expanded UC MSCs, their surface protein expression at p4 and subsequent passages was next investigated. Phenotypic characterization was performed at four different passages (representing approximately 17, 19, 21, 25 PDs). MSC-specific markers CD90, CD73 and CD105\(^3\) were uniformly positive showing a stable expression profile throughout the expansion, whereas the absence of EC marker CD31 showed no contamination with ECs (Figure 1A).

Despite the expression of NGFR transcript, CD271 surface protein expression was absent in culture-expanded MSCs at all tested time-points (Figure 1A). CD146 expression declined gradually and correlated with telomere loss in the two cultures tested (Figures 1A and 1B).

Telomere length measurement in sorted CD146\(^-\) and CD146\(^+\) populations
The cell sorting strategy for these experiments is described in Figure 2A. Following FSC/SSC gating to define cells (R1), live cells (R2) containing distinct populations of ECs (R3) and HCs (R4) were clearly observed. In general, HCs were most abundant (mean 48\% of total live cells, n=10). CD31\(^+\) ECs represented a mean of 1.4\% of live cells. Double negative cells (R5) were further subdivided into CD146\(^-\) (R6, candidate UC MSC) and CD146\(^+\) (R7, non-MSC) subsets and sorted into separate RNA lysis buffers. The yields of sorted cell subsets are shown on Figure 2B, left panel.

Within the double-negative cells, the CD146 population was predominant over the CD146\(^+\) population (mean 81\% and 17\% respectively, this effect was not evident for CD90 which remained stable (Figure 1B). When comparing the phenotypic profile of UC MSCs with ECs only CD31 revealed high specificity for ECs; CD73, CD105, CD146 were expressed on both UC MSCs and ECs (data not shown).

In combination with the molecular profile of UC MSCs and the previously-published literature\(^5\), these data indicated that uncultured UC CD146\(^+\) cells could indeed possess longer telomeres than the remaining CD146\(^-\) cells.

## Table 1. Relative gene expression in UC MSCs compared to skin fibroblasts.

<table>
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<th>Gene</th>
<th>TaqMan assay ID</th>
<th>Description</th>
<th>UC MSCs</th>
<th>FIBs</th>
<th>UC MSCs to FIBs</th>
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<td>NGFR</td>
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<td>1.23</td>
<td>2.41</td>
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<td>NANOG</td>
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<td>Nanog homeobox</td>
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<td>0.03</td>
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Figure 1. MSC marker expression in culture-expanded UC MSCs. A - Surface expression levels of MSC markers (n=3 donors, y error bars indicate SD). B - Donor variation of CD146 and CD90 markers in early (<20 PDs) and late (>20 PDs) cultures (M1: marker expression). Bottom panels - telomere T/S ratios were directly correlated to the expression levels of CD146 and not CD90 during culture-expansion. p<0.05; Figure 2A). When these frequencies were re-calculated in relation to total live cells, the CD146+ and CD146- fractions represented a mean of 10.4% and 37.7%, respectively (Figure 2B, right panel). This was significantly higher than the frequency of CFU-Fs (as a percentage of total live cells) (mean 0.01%, Figure 2B, right panel). When the telomere length of both sorted subsets (CD146+ and CD146-) were tested, the CD146+ subset exhibited higher telomere lengths compared to the CD146 subset in five out of seven donors (Figure 2C). The median difference suggested that CD146+ cells’ telomeres were 28bp longer (range 6414–7187bp) compared to CD146- cells (range 6393–7120bp); however, the observed differences failed to reach statistical significance. Interestingly, the T/S ratio of the candidate MSC population falls closer to those of the adult tissues (BM MSCs and endometrial cells) than foetal tissues described by Guillot et al.21.

The high frequency of CD45-,CD31-,CD235α-,CD146+ cells compared to the CFU-F frequency and the lack of consistent and significant enrichment for cells with long telomeres indicated that the CD146+ UC fraction most likely contained non-haemopoietic cells with varying degrees of maturity.

CD146 expression in UC tissue in situ
Initial haematoxylin staining (Figure 3A) revealed the basic structure of the UC tissue where the UC vein and artery could be seen. This was surrounded by the single thickness endothelial area (EA) and highly organised muscular perivascular area (PA). The Wharton’s jelly (WJ) matrix could also be seen spanning intra-muscular areas.

Immunohistochemistry was next used to investigate marker expression in situ including semi-quantitative assessment in different anatomical areas (Figures 3B and 3C). Three optical microscope fields (>200) were evaluated per anatomical area of UC tissue. CD271 was present at low levels in the WJ area only. EC-specific CD31 and CD34 were present in the EA of the vessels. The intracellular marker of smooth muscle cells; αSMA22 was expressed and had its highest positivity in the PA surrounding vessels. CD90 and CD146 were highly expressed in most UC compartments, including the PA; however CD146 but not CD90 showed positivity for the EA area. This was consistent with previously published literature9. Representative photomicrographs are shown in Figure 3C.

Overall, our immunohistochemistry results revealed the expected topography of CD31+ and CD34+ ECs, the expression of CD271 in the WJ area, and the preferential topography of CD90 and αSMA in PA. Consistent with previous findings9, CD146 was expressed in WJ, PA and EA, with the highest proportion of cells present in the PA. Wide distribution of CD146+ cells in all anatomical areas of UC tissue was consistent with the high frequency of CD45-CD31-CD235α-CD146+ cells evident by flow cytometry. This indicated that UC MSC isolation alone, even after the removal of CD31+ ECs, was not sufficient to purify native MSCs from the UC tissue.
Figure 2. Sorting strategy and telomere length measurements in putative native UC MSCs. A - Cell sorting strategy: nucleated cells (upper left graph, R1) were gated based on FSC/SSC profile; live cells (upper right graph, R2) were identified by 7AAD exclusion method. On average, nucleated cells (R1) represented 27% of total events collected whereas excluded events corresponded to red blood cells and cellular debris. Following R2 gating, three distinct populations were evident: middle right graph, R3/ECs, R4/HCs, and R5/double-negative. Gating on double-negative subset (R5) revealed two subsets (bottom right graph), CD146⁺ (R6/candidate MSCs) and CD146⁻ (R7/non MSCs); isotype control staining is shown on the bottom left panel. Cells confined to regions R6 and R7 were sorted and processed for telomere length analysis. B - The total yields of CD146⁺ and CD146⁻ fractions (left panel) and their percentage of total live cells compared to the percentage of CFU-Fs (right panel, n=10 donors for sorted subsets, n=6 donors for CFU-F). Box and whiskers plots represent quartiles and range respectively, bar indicates median, y error bars indicate SD, *p<0.05, **p<0.01, ***p<0.001. C - Telomere T/S ratios in sorted subsets, normalised according to Cawthon 2002 (n=7 donors).
Figure 3. Tissue architecture of UC vein, artery and surrounding Wharton’s jelly. A - Images show endothelial area (EA, indicated by arrows), perivascular area (PA, multiple layers of muscle fibres) and Wharton’s jelly area. B - Expression of MSC and EC markers in UC tissue. C - Staining of MSC and EC markers in UC tissue (representative donor and cross sections; (+) symbol indicates the expression of a marker and (-) symbol indicates the absence of expression of a marker).

**Dataset 1. Version 2. CD146-based cell sorting and telomere length in umbilical cord**

http://dx.doi.org/10.5256/f1000research.4260.d34848

UC MSC and fibroblast cultures were evaluated for their expression levels of selected transcripts characterizing multiple cell fates in vivo. Ct values obtained using Taqman qPCR technology are shown in dataset a (UC=Umbilical cord; FIB=fibroblast cell lines). The flow cytometry phenotype profiles of UC MSCs (cultures tested against a panel of MSC markers) and UC ECs (cultures tested against a panel of EC markers) are shown in dataset b. Telomere lengths of cultured UC MSC1 and MSC2 samples are displayed in dataset c (telomere lengths calculated as T/S ratio in different passages of UC MSC cultures). Telomere lengths as T/S ratio and calculated length of freshly sorted cells are shown in dataset d. Telomere length was calculated as T/S ratio and length in bp from the freshly sorted CD45 CD235a CD31 CD146⁻ (candidate MSCs) and donor-matched CD45 CD235a CD31 CD146⁺ (non-MSCs) of n=7 UCs.

**Discussion**

The present study assessed the possibility of using CD146-based cell sorting and telomere length analysis for establishing the identity of mesenchymal stem cells in human UC. Previous studies have shown that the UC CD146⁺ subset contained MSCs able to differentiate into osteoblasts, chondrocytes and adipocytes. Other studies have shown that ‘true’ immature stem cells have much longer telomeres compared to the remaining mature cells. Here we tested whether CD146⁺ UC cells, that were depleted of contaminating haemopoietic and endothelial cells, had longer telomeres compared to the corresponding CD146⁻ population.

Initially we performed gene expression analysis, flow cytometry and telomere length measurements on culture-expanded UC MSCs during their extensive passaging. The mesenchymal tripotentiality of these cultures has been demonstrated in our previous study. In this study, gene expression analysis revealed that MCAM/CD146
was expressed at higher levels (>3-fold) in UC MSCs compared to fibroblasts, confirming its potential specificity for MSCs. Whereas common MSC-specific markers CD73, CD105, CD90\(^{40}\) displayed stable expression throughout passaging, CD146 expression declined with the increased number of cell divisions at later passages consistent with a loss of multipotent progenitors\(^{1}\). The CD271 surface marker was not expressed at any stage during UC MSC cultivation, consistent with the loss observed in BM MSCs\(^{34,42,45}\). Although CD271 is expressed on uncultured BM MSCs, it was not selective for UC blood MSCs\(^{19,26}\). Therefore, CD271-based MSC isolation from UC tissue was not pursued further. However, the decrease in CD146 during expansion correlated with telomere erosion in the same cultures, supporting the idea that CD146 could mark the most immature cells \textit{in vivo}. Based on the present findings and previous data\(^{4}\), CD146 was selected as a candidate positive marker for sorting native MSCs from UC digests. CD45/CD235a and CD31 were used to exclude native HCs and ECs from the analysis, respectively\(^{4}\).

Sorted CD146\(^{+}\) and CD146\(^{-}\) subsets were next compared with respect to their telomere length. Several previous studies provided initial evidence that UC MSCs express telomerase continuously and hence were able to retain long telomeres\(^{27,28}\). In UC blood MSCs, a distinct SSEA-4\(^{-}\)/CD105\(^{-}\)/MSCA-1/CD90\(^{-}\) cell population was shown to have longer telomeres than the SSEA-4/CD105\(^{-}\)/MSCA-1\(^{-}\)/CD90\(^{-}\) subset\(^{25}\). Although evident in five out of seven experiments in our study, there was no significance to the difference in telomere length between CD146\(^{+}\) and CD146\(^{-}\) subsets potentially indicating contamination of MSCs with more mature cells in the CD146\(^{-}\) subset. The high frequency of CD146\(^{+}\) cells (10.4%) was in stark contrast to the CFU-F frequency (~0.01%), which was consistent with previous findings\(^{3,5}\). Furthermore, the immunohistochemistry data confirmed the broad reactivity of CD146 with UC tissue anatomical areas\(^{4}\). Altogether, these data provided the first indication that the CD146\(^{+}\) population was unlikely to represent pure native UC MSCs.

Alternatively, a lack of significant difference in telomere lengths could be explained by the limited sensitivity of qPCR assay to measure telomere lengths\(^{31}\). Telomere length assessment in the majority of studies using cultured MSC is based on terminal restriction fragment (TRF) analysis\(^{32–36}\), which has the disadvantage of producing a smear of bands rather than a discrete band/point affecting accurate quantifications. Additionally, the large number of cells required for such analysis precluded its use on sorted UC cells. On the other hand, studies investigating the correlations between telomere lengths derived by qPCR and TRF indicated good levels of correlation (r>0.823)\(^{37}\), although qPCR was shown to be limited in its ability to measure the longest telomeres\(^{31}\). This potential technical limitation of qPCR, as well as working at the lower limit of DNA concentrations, could have affected the accuracy of the telomere analysis in the present study.

Our findings could be further compounded by the large donor-to-donor variation, which was evident in culture expanded UC MSCs (~10% between donor 1 and 2, Figure 2B), but also with sorted subsets (Figure 2C). One recent study has demonstrated that telomere lengths could be heritable, with the parental age at conception being a factor affecting offsprings' telomere length in leukocytes (LTL)\(^{38}\). Although the mode of LTL inheritance has been suggested to be X-linked\(^{33}\), another study shows a paternal mode of heritability\(^{39}\). The parental ages of the UC donors were not known/recorded in this study but would be an interesting subject for the future work. Previous studies have suggested that the magnitude of inter-individual variation in telomere lengths could exceed the variation between cell types within the same individual\(^{10–12}\), therefore telomere length measurement approaches should be further refined before they can be used as a tool to identify MSCs \textit{in vivo}.

To conclude, the broad availability of UC tissue makes it amenable to be used in cell therapy and regenerative medicine interventions. To this end, the present study showed that in the UC, CD146\(^{+}\) cells were too numerous to be selective for pure native MSCs and were likely to contain more mature cells. Therefore, additional markers would be needed to isolate MSCs from UC tissue.

**Data availability**


**Consent**

Written informed consent has been obtained from full-term caesarean section patients for the use of clinical samples in the present study.

**Author contributions**

DK, SMC, DM, EJ conceived the study. DK performed the experimental work. All authors were involved in manuscript preparation and have agreed to the final content.

**Competing interests**

No competing interests were disclosed.

**Grant information**

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\textit{The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.}

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Open Peer Review

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Simone Pacini
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Authors addressed any queries and the revised manuscript clarifies all the aspects that were originally ambiguous, in the first version of the paper. Thus I believe that the manuscript, in this form, fulfills the scientific standard requirements.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 23 July 2014

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Simone Pacini
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I carefully read the manuscript entitled “The assessment of CD146-based cell sorting and telomere length analysis for establishing the identity of mesenchymal stem cells in human umbilical cord” from Kouropis et al. The aim of the study is verifying the hypothesis that umbilical cord-derived MSCs
reside in the CD146-positive fraction, and could be discriminated by their possible longer telomeres. The authors demonstrated that frequency of CD146-positive cells in the culture-initiating suspension was much higher than the frequency of CFU-F confirming that this population also include non MSC-progenitors. Telomere length analysis revealed non-significant differences between CD146-positive and -negative cells, in three of four samples. Thus, the authors correctly conclude that the CD146-positive population was unlikely representing pure native UC-MSCs, and that a prospective isolation of ex vivo MSC necessarily should include more antigens to investigate.

My review will be concentrated on some technical aspects regarding conduction of cell sorting and telomere analysis, which should be clarified. My major comments are as follows:

- From the density plots reported in figure 1A, it is not clear if a morphology gate was applied in the SSC/FSC plot, to exclude events of no-cellular origins. A Gate was applied in the dead-cells exclusion plot and in R1 would be counted no-cellular 7-AAD negative events, which could significantly alter the CD146 positive or negative percentages on total living cells. The authors extensively discussed the percentage of CD146-positive frequency in correlation to CFU-F frequency. Thus, the authors should clarify this aspect, and I strongly suggest clearing the flow cytometry data from the non-cellular events.

- As the CD146-positive and -negative populations are not sufficiently separated in the R4 gate, the sorting gates R5 and R6 should be defined by applying isotypic control. Moreover, I suggest reporting the isotypic control plot in the figure, in order to demonstrate the absence of events in the R5 region.

- Purity, and possible Yield, of sorted cells should be indicated in the results section, in order to reassure the reader of the quality of the sorting process.

- Telomere length has been described as T/S ratio, reported data are close to the value of 1.0 and two of four have values lower than 1.0. How could it be possible to detect telomere repeat copy numbers lower than the single copy gene (36B4)? According to Cawthon R.M. (2002) T/S ratios should be normalized. PCR efficiency could vary gene to gene, and should be evaluated in order to normalize qPCR data with the efficiency correction factor.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Aug 2014

**Dimitrios Kouroupis**, Foundation for Research and Technology-Hellas, Institute of Molecular Biology and Biotechnology, University of Ioannina, Ioannina, Greece
“From the density plots reported in figure 1A, it is not clear if a morphology gate was applied in the SSC/FSC plot, to exclude events of no-cellular origins. A Gate was applied in the dead-cells exclusion plot and in R1 would be counted no-cellular 7-AAD negative events, which could significantly alter the CD146 positive or negative percentages on total living cells. The authors extensively discussed the percentage of CD146-positive frequency in correlation to CFU-F frequency. Thus, the authors should clarify this aspect, and I strongly suggest clearing the flow cytometry data from the non-cellular events.”

We are very grateful to Dr. Pacini for his careful reading of our work and for his insightful comments. We believe that Figure 1A that he refers to is in fact Figure 2A as this pertains to our cell sorting experiments from umbilical cord (UC) tissue digests. We can confirm that we did perform FSC/SSC gating to remove anuclear red cells and cellular debris in all our sorting experiments. It was an oversight that this was omitted in the original submission and we thank the reviewer for highlighting this. We have now modified the relevant section of our Materials and Methods as well as Figure 2A to reflect this (gate R1). Additionally, we have added average percentage of cells within R1 out of all events collected into the appropriate Figure Legend.

“As the CD146-positive and -negative populations are not sufficiently separated in the R4 gate, the sorting gates R5 and R6 should be defined by applying isotypic control. Moreover, I suggest reporting the isotypic control plot in the figure, in order to demonstrate the absence of events in the R5 region. Response: We appreciate this comment. In our experiments with UC tissue digests, a distinct CD45-CD235a-CD31-CD146+ population was always evident above the isotype control staining.”

We further acknowledge that our original density plots failed to show that our sorting gates were always set apart to ensure maximal sorting purity. We have made new flow cytometry plots for panel 2A to reflect all of these changes (FSC/SSC gating and isotype control panel).

“Purity, and possible Yield, of sorted cells should be indicated in the results section, in order to reassure the reader of the quality of the sorting process.”

We have now provided information on CD146+ and CD146- fractions’ cell yields in Figure 2B, left panel. We acknowledge that we did not make clear in our original submission how many sorting experiments we performed in total, we confirm that this is ten for the sorting experiments. Fraction purities post-sort are always >95% using our cell sorter (see Churchman et al., 2012).

“Telomere length has been described as T/S ratio, reported data are close to the value of 1.0 and two of four have values lower than 1.0. How could it be possible to detect telomere repeat copy numbers lower than the single copy gene (36B4)? According to Cawthon R.M. (2002) T/S ratios should be normalized. PCR efficiency could vary gene to gene, and should be evaluated in order to normalize qPCR data with the efficiency correction factor.”

We appreciate this comment and agree that it is indeed a valid point that we should clarify further. Our method was based upon that of Cawthon R.M. (2002) as stated...
above and in the text, however when using unexpanded cells sorted from UC the cell numbers were limited because of the amount of tissue processed and inevitable cell losses caused by multiple centrifugation steps prior to cell sorting. Due to this we elected to standardise our telomere length experiments to 1000 sorted cells per fraction (previously overlooked from methods). We accept that we were working at the lower limit of DNA concentrations (acknowledgement of this is now added as a new sentence in Discussion), but believe that with triplicate replicates and extremely comparable standard curves (prepared using immortal cell lines) on each plate for inter-plate comparisons, our work is technically sound, and that our standardisation and technical control was suitable for comparing the differences between the CD146+ and CD146- populations. We did not normalise our T/S ratios and chose to show the data as a direct comparison of sample pairs and therefore some of these values fell below 1. This was also observed in the Cawthon paper; a ‘raw’ T/S ratio of 0.69 is what he normalised against to give the lowest value of 1 (Figure legend 5). Values below 1 can also be observed in Guillot et al. (2007). We have added an interesting observation that our umbilical cord T/S ratios fall closer to the adult tissues than the foetal tissues in that study. We now present normalized T/S ratios adjusted according to Cawthon R.M. (2002) in Figure 2C and conversion to telomere length in the text. We have also strengthened this figure with new data from a further 3 sorting experiments (new total n=7) and have performed statistical analysis. The CD146+ fraction possesses telomeres with median value 28bp longer than the CD146- fraction, although this was not significant (Wilcoxon).

**Competing Interests:** No competing interests were disclosed.

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Armin Attar
Shiraz University of Medical Sciences, Shiraz, Iran

In the current study, the investigators have used cell sorting techniques as well as telomere length analysis to assess the identity of UC tissue derived mesenchymal stromal cells. The manuscript is scientifically sound and deserves indexation.

**Competing Interests:** No competing interests were disclosed.
I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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