Aging Bone Marrow Mesenchymal Stromal Cells Have Altered Membrane Glycerophospholipid Composition and Functionality

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† Abbreviations: AA, arachidonic acid; CFSE, 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester; CoA-IT, CoA-independent transacylase; COX, cyclooxygenase; cPLA2, cytosolic phospholipases A2; DAG, diacylglycerol; DHA, docosahexaenoic acid; FA, fatty acid; GPL, glycerophospholipid; GO, gene ontology; hBMSC, human bone marrow mesenchymal stromal cell; hMSC, human mesenchymal stromal cell; LOX, lipoxygenase; miRNA, micro ribonucleic acid; MSC, mesenchymal stromal cell; MUFA, monounsaturated fatty acid; PBMC, peripheral blood mononuclear cell; PC, phosphatidyl choline; PE, phosphatidylethanolamine; PG, prostaglandin; PGE2, prostaglandin E2; PI, phosphatidylinositol; PS, phosphatidylserine; SFA, saturated fatty acid, TRF, terminal restriction fragment
Abstract

Human mesenchymal stem/stromal cells (hMSCs) are increasingly used in advanced cellular therapies. The clinical use of hMSCs demands sequential cell expansions and since it is well established that membrane glycerophospholipids (GPLs) provide precursors for signalling lipids that modulate cellular functions, we studied the effect of the donor’s age and cell doublings on the GPL profile of human bone marrow MSC (hBMSC). The hBMSCs, harvested from 5 young adult and 5 old donors, showed clear compositional changes during expansion, seen at the level of lipid classes, lipid species and acyl chains. The ratio of phosphatidylinositol to phosphatidylserine increased towards the late passage samples. Furthermore, 20:4n-6 containing species of phosphatidylcholine and phosphatidylethanolamine accumulated while the species containing monounsaturated fatty acids (FAs) decreased during passaging. Additionally, in the total FA pool of the cells 20:4n-6 increased, which happened at the expense of n-3 polyunsaturated FAs, especially 22:6n-3. The GPL and FA correlated with the decreased immunosuppressive capacity of hBMSCs during expansion. Our observations were further supported by alterations in the gene expression levels of several enzymes involved in lipid metabolism and immunomodulation. The results show that extensive expansion of hBMSCs harmfully modulates membrane GPLs, affecting lipid signalling, and eventually impairing functionality.

Supplementary keywords: glycerophospholipid profile, mesenchymal stem cell, arachidonic acid, docosahexaenoic acid, aging, lipid signalling, mass spectrometry
Introduction

Human mesenchymal stem/stromal cells (hMSCs) are currently being studied in a number of clinical applications, for example to improve the engraftment of hematopoietic stem cell transplant, promote myocardial repair and control immunological responses in graft versus host disease, autoimmune diseases and solid organ transplantations (1-5). In addition to being immunologically privileged, these cells can modulate both innate and adaptive immune responses both in vitro and in vivo. hMSCs have been shown to be able to inhibit T-cell proliferation, inhibit dendritic cell maturation (6), recruit regulatory T-cells (7,8) and also to modulate B-cell functions (9). The mechanisms by which these cells exert their immune modulatory functions are still unclear, but it is likely that both direct cell-cell contacts and the secretion of soluble factors are needed. Several cytokines, growth factors, enzymes and lipid mediators, such as transforming growth factor β1, the tryptophan degrading enzyme indoleamine 2, 3-dioxygenase and prostaglandin E2 (PGE₂), have been indicated as key players in the immunomodulatory process (7,10,11).

A long expansion period has been considered to have an adverse effect on the proliferation and differentiation potential and other functional properties of progenitor cells (12,13). One of the key questions in MSC therapy is: how many cell doublings the cells can undergo before the risks of cellular malfunction or even malignant transformation surface? The number of safe cell doublings may be dependent on the cell source, donor age and culture conditions.

To date, only a few studies on stem cell membrane lipids have been performed (13,14) and the changes in lipid composition during the expansion have not been studied systematically. Although glycosphingolipids and signalling lipids have been studied in relation to the cell identity and functions (15-18) an important group of membrane lipids, the glycerophospholipids (GPL), composing the main part of membrane lipids, have received less attention. The most prominent class, phosphatidylcholine (PC), makes up approximately one half of all membrane phospholipids. Phosphatidylethanolamine (PE), -serine (PS) and –inositol (PI) are the other common mammalian GPL classes. Each class consists of numerous different molecular species with different acyl, alkyl or alkenyl chain assemblies (19). Consequently, a single cell contains more than a thousand different GPL molecular species (20,21). However, the ultimate reasons for the structural diversity of cellular lipids, apparently of a great functional importance, are still largely unknown. Modern mass spectrometry and bioinformatics tools offer new ways to analyse the compositional changes in complex stem cell lipidomes and link those with the altered potency and quality of the cells (22-24).
Membrane lipids not only form a protective layer around the cell but also mediate biological signals through several pathways employing G-protein activators, second messengers, and nuclear receptor activators (25,26). The signalling pathways utilizing phosphorylated PIs (PIPs), eicosanoids, or sphingolipids are well understood. Cellular mediators, such as inositol phosphates, diacylglycerols (DAGs), lysosphospholipids, ceramides, cleaved fatty acids (FAs) e.g. arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), or their derivatives are released from membrane lipids and play an important role in many physiological processes including the regulation of inflammation. The essential polyunsaturated fatty acid (PUFA) 20:4n-6 is found primarily at the sn-2 position of most membrane phospholipids. It is a precursor for the synthesis of prostaglandins (PGs), tromboxanes and leukotrienes (27), one of which, a specific PGE\textsubscript{2}, has been proposed as one of the soluble factors mediating the MSC immune function (7,28) The n-3 PUFAs, especially 22:6n-3, are precursors for resolvins, protectins and maresins which, in contrast to 20:4n-6 and most n-6 PUFAs, exert their functions during the resolution phase of inflammation (29).

The aim of our study was to compare the GPL profiles of hBMSCs from young and old donors, study the effects of sequential expansion of the cells on these profiles, determine the expression of genes related to lipid metabolism and immunomodulation, and gain a better understanding of the possible relations of cell lipidome changes and functionality. Knowledge of the connections of lipid alterations during expansion and their effect on the immunosuppressive capacity of the therapeutic cells may have a profound influence on clinical cell expansion protocols.
Materials and methods

Ethics and bone marrow donors
All patient protocols were approved by the Ethical Committee of Northern Ostrobothnia Hospital District or Ethical Committee of Hospital District of Helsinki and Uusimaa. The hBMSCs were obtained from bone marrow aspirates taken from the iliac crest or upper femur metaphysis of adult patients after written informed consent. The hBMSCs from donors of different ages (anonymous coding) were isolated as previously described (30,31).

Cell culture of hBMSC
The cells were cultured in minimum essential alpha-medium (αMEM) supplemented with 20mM HEPES, 10% fetal bovine serum (FBS), 2mM L-glutamine and 100 units/ml penicillin and 100 μg/ml streptomycin (all from Gibco, Invitrogen, Paisley, UK). The same serum lot was used throughout the study. The medium was renewed twice a week, the cells were harvested when 70-80% confluent and plated at a density of 1000 cells/cm². Population doublings for every passage were calculated using the formula NH=2^{PD} x N1, where NH is the number of cells harvested and N1 is the number of cells plated. For lipid analysis, the cells were washed with ice-cold PBS scraped on ice pelleted in silylated vials and stored at -70°C for later use. The expression of surface antigens CD13, CD14, CD19, CD29, CD34, CD44, CD45, CD49e, CD73, CD90, CD105, CD106, CD166, CD271, HLA-ABC and HLA-DR were analyzed by flow cytometry (FACSAria Becton Dickinson, San Jose, CA USA and FlowJo 7.6.1 software Treestar, Asland, OR, USA) (Table S1).

Phospholipid mass spectrometry and fatty acid gas chromatography
Total lipids of the hBMSCs were extracted according to Folch et al. (32), spiked with internal standards, and dissolved in chloroform/methanol 1:2 for direct infusion experiments. Several internal standards were used to correct for the effects of the polar head group and acyl chain length on the instrument response according to previously reported procedures (33-35). Just prior to the mass spectrometry analysis, 1% NH₄OH was added and the lipid extracts with the internal standards were infused to the electrospray source of a Quattro Micro triple quadrupole mass spectrometer (Micromass, Manchester, UK) at the flow rate of 8 μl/min. The collision energy of the instrument was set to 25–65 eV, and negative and positive ion modes were used. Argon was used as the collision gas. The PC and lysoPC (precursor of 184), PE (neutral loss of 141), PS (neutral loss of 87), PI (precursor of 241) species were selectively detected using head-group specific MS/MS scanning modes (36,37). The acyl chain assembly of the major lipid species was confirmed by MS/MS techniques. The mass spectra were processed by MassLynx software (Micromass, Manchester, UK) and the individual lipid
species quantified by using the internal standards and LIMSA software (38). The lipid species were abbreviated: [total carbon number in the chains]:[total number of double bonds in the chains]. The relative concentrations of the lipid classes were obtained by summing up the concentrations of the individual molecular species in a class.

The FAs in the total lipids of the cells were determined as methyl ester derivatives by gas chromatography as detailed in Käkelä et al. (39). The FA composition was calculated as mol%, and the FAs were marked by using the abbreviations: [carbon number]:[number of double bonds] n-[position of the first double bond calculated from the methyl end] (e.g. 22:6n-3).

**Co-culture assay**

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy anonymous blood donors (Finnish Red Cross Blood Service) by density gradient centrifugation (Ficoll-Pague plus, GE Healthcare, Piscataway, NJ, USA) and cryo-preserved for later use. Prior to use, the PBMCs were thawed gently and labeled with 5 µM CFSE (5(6)-Carboxyfluorescein diacetate N-succinimidyl ester) solution (Molecular probes, Oregon, USA). The CFSE-labeled PBMCs were cultured in triplicates at 1.5x10^6 cells/well in a 48-well plate with hBMSCs in RPMI growth medium (RPMI, 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin). hBMSCs were allowed to attach for two hours before PBMCs were added. To activate the T-cell proliferation, 100ng/ml of antihuman CD3 antibody clone Hit3a (BioLegend, San Diego, CA, USA) was added to the co-culture. T-cell proliferation was recorded after four days of incubation as a dilution of fluorescent dye by flow cytometry.

**Telomere length analysis**

Telomere length were analyzed by the southern blot analysis of terminal restriction fragment (TRF) lengths (40) Genomic DNA from snap-freezed cell pellets was purified using the Qiagen DNeasy Blood and Tissue Kit and extracted with ethanol. Quality of purified DNA was evaluated by 1% agarose gel electrophoresis. Telomere length analysis was performed using TeloTAGGG Telomere Length Assay Kit (Roche, Basel, Switzerland). DNA was digested using RsaI and HinfI enzymes and electrophoresed on a 0.8% agarose gel 5V/cm. Southern blotting was performed using 20X salium sodium citrate buffer (SSC). The blot was hybridized overnight using a digoxigenin (DIG)-labeled telomere specific probe (TTAGGG) and incubated with alkaline phosphatase-labeled anti-DIG antibody. The blot was then incubated with CDP-Star chemiluminescent substrate and exposed to autoradiography film (GE Healthcare). The autoradiogram was scanned by densitometry and TRF length was
calculated using ImageJ analysis software (41) according to \( TRF = \frac{\Sigma OD}{\Sigma (OD/L)} \), where \( OD_i \) is optical density and \( L_i \) is the length of the TRF at position \( i \). TRF signals between 3 and 20 kb were used for telomere length measurements (40).

**Western blotting**

Snap-freezed cell pellets were lysed using RIPA–buffer (Thermo Scientific, Rockford, IL, USA) containing Protease Inhibitor Cocktail (Sigma, St.Louis, MO, USA). Protein concentrations were determined using BCA protein assay kit (Pierce, Rockford, IL USA). 20 \( \mu g \) of total protein was run on a 12% SDS-PAGE gel (Bio-Rad, Laboratories Hercules, CA, USA) and electrotransferred to Hybond ECL Nitrocellulose membrane (GE Healthcare). The membrane was then blocked with 5% milk in PBS containing 0.1% Tween-20 and immunoblotted using anti-p16\(^{INK4A} \) (1: 800, clone DCS-50) (Sigma)) and anti-p21 (1:250, Clone SXM30) (BD Pharmingen, USA). \( \beta \)-actin (1: 8000, monoclonal anti-\( \beta \)-actin, clone AC-74) was used as a loading control (Sigma)). Polyclonal anti-mouse horseradish peroxidase (HRP) conjugated antibody was used as a secondary antibody (1:1000) (Dako Cytomation, Glostrup, Denmark). Detection was performed using enhanced chemiluminescent detection system (ECL, GE Healthcare). Quantification of band intensities were performed using GS 800 densitometer and Quantity One software (both from Bio-Rad Laboratories)

**Microarrays**

RNA was extracted using Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, CA, USA) and a Qiagen supplementary protocol “Purification of total RNA containing miRNA from animal cells using the RNeasy Plus Mini Kit “according to the vendor’s instructions. Extracted DNA was stored for later use and not used in this study.

Labeled RNAs (800 ng/sample) were hybridized onto Agilent SurePrint G3 Human GE 8x60K and then the slides were washed and scanned according to the manufacturer’s recommendations. The raw data files (.txt files) were imported into the R v. 2.13 software (42) and preprocessed by the BioConductor package limma v.3.4.5 (43). After quality control of the data, the median probe intensities were log2 transformed and normalized according to the method of the quantiles (44). The probes for the same Entrez Genes or lincRNAs (as of 1st January 2012) were averaged.

A linear model including the AGE*PASSAGE + SUBJECT + DYE terms followed by a moderated t-test was utilized for finding the differentially expressed genes (log2 Fold Change >0.58) in the comparisons of interest (nominal p-value < 0.01). The differentially expressed
genes were illustrated with violin plot by using R package vioplot with height argument 0.4 and analyzed for significant enrichments of GO-BP classes by using R library GOSim v. 1.2.5 (45). Enrichments with a p-value <0.01 were considered significant. Default parameters were used in the GO analysis.

**Statistical analysis**

The data was analysed both in univariate and multivariate way. In the univariate approach, each GPL species or individual FA was analysed separately and presented in figures as a mean ± standard deviation. Statistical significances for the differences between hBMSCs from the young and old donors or early and late passages were calculated using student’s paired t-test (* p-value <0.05, ** p-value< 0.01 an p-value <***0.001). In addition, the changes in each lipid percentage were analysed with linear mixed effects models, fitting a model with fixed terms for age and passage and a random effect for cell line (a repeated measures type analysis). The effect of each term for every lipid was estimated using normal F-test p-values and visualized with interaction plots depicting the mean values in each passage and group. Software for statistical analysis was computing language R, version 2.12, using package “nlme” for a mixed effects analysis.

In the multivariate approach the lipidome data were subjected to Principal Component Analysis (PCA) to study differences between the samples in terms of the whole GPL profile, and to find out which GPL species were mainly responsible for the variation in the data. PCA was computed using log10 normalized data and the relative positions of the samples and variables were plotted using the first two principal components. In addition, quantitative multivariate measures of the differences among the sample groups were determined by Soft Independent Modelling of Class Analogy (SIMCA) (46).
Results
Cultivated hBMSCs reached replicative senescence, and the cells from old donors showed larger variation in their growth potential

hBMSCs isolated from five elderly (from 62 to 82 years of age, mean 74.6 years) and five young adult donors (from 20 to 24, mean 22.2 years) were characterized, and shown to fulfil the established minimal criteria for MSCs (47) (Figure S1). At the end of the cell culture (after 45 to 108 days; depending on the donor), the hBMSCs adopted typical senescent morphology (“fried egg”; Figure 1A, right). The proliferation capacity of the primary hBMSCs was assessed by determining the cumulative population doublings as cells were passaged until senescence. The hBMSCs from old donors showed greater individual variation in their growth kinetics than those from young donors (Figure 1B). It should be noted that the cell doublings before passage 4 were not included in the calculations. The replicative senescence was further studied from the selected samples and passages. The replicative senescence enhanced the expression of cell cycle components p16\(^{\text{INK4A}}\) and p21\(^{\text{CIP1/WAF1}}\) (Figure 1C). Additional proof was obtained from telomere length measurements. Telomere length in passage 11 cells was 1.0 ±0.5 kbp shorter than at passage 4 (Figure 1D, Figure S2).

GPL profiles of hBMSCs from young and old donors

The average values for GPL class totals for hBMSCs were: PC 41-46%, PE 34-38%, PS 5-8% and PI 4-8% (Table 1). In general, the hBMSCs from the young and old donors had very similar class profiles. The GPL species of the hBMSCs (Figure 2) showed that the major PC species (in descending order) were 34:1, 36:2, 36:1 and 38:4 (Figure 2A), while the most abundant PE species were 38:4, 36:1, 36:2, 40:5, 38:5 and 34:1 (Figure 2B). The predominant PS species were 36:1, 40:5 and 40:6 (Figure 2C), and a single species 38:4 accounted for almost 50% of the total PI (Figure 2D). In the early passage samples, statistically significant differences (p-value<0.05) due to the donor age were found for PC38:5 and PE38:4 (Figures 2A,B). The levels of PE alkenyl and PC alkyl species also altered during the long-term passaging (Table 1).

Expansion of hBMSCs increases membrane PI content in relation to PS

During long-term cultivation, PI totals increased especially in the cells from young donors (Table 1). This increase of PI became even clearer when the PI totals were studied in relation to the PS totals. In the hBMSCs from the old donors, the PI/PS ratio was already high at the earliest passage (Figure 3). Interestingly, also lysoPC totals increased consistently during the cell expansion of the hBMSCs from young donors but showed varying responses in the hBMSC from old donors (Figure S3).
Expansion of hBMSCs increases 20:4n-6 containing GPL species at the expense of MUFA and n-3 PUFA containing species

Expansion of the hBMSCs induced alterations in the relative amounts of numerous individual lipid species, as revealed by ESI-MS. For instance, in the PC diacyl species the mol% of 38:4, 36:4 and 36:1 increased towards the latest passages, while the mol% of 34:1, 34:2 and 36:2 decreased (Figure 4 A, B). The PE diacyl species profiles exhibited an increase in 38:4 and 36:4 species and a decline in the monounsaturated species (34:1 and 36:1) and in those containing n-3 PUFAs (40:5, 40:6 and 40:7). The most dramatic change was seen in the PC and PE diacyl species 38:4. Over all the passages, the levels of PC34:1 and PE36:1 species in the hBMSCs from the young donors were consistently higher than those from the old donors (Figure 4C). These changes were further highlighted when multivariate statistical analysis (PCA) indicated the 38:4 and 36:4 species of PC and PE (with 20:4n-6) as the ones explaining the greatest part of the compositional variation among all the studied hBMSCs (Figure 4D). In the PCA biplot, these 20:4n-6-containing species had an inverse relationship with the mono- and diunsaturated ones. The principal component 1 explained up to 52% of the variation among the samples.

The FA profiles by GC showed that the proportions of individual saturated FAs (SFAs), such as 18:0, increased towards the late passages whereas individual monounsaturated FAs (MUFAs) decreased (Figure 5A). Among n-6 PUFAs, 20:4n-6 increased clearly and constantly throughout the expansion along with 20:3n-6, while other principal n-6 PUFAs decreased (Figure 5A, S4). At the same time, the two prominent n-3 PUFAs, 22:5n-3 and 22:6n-3, decreased, the response being clearer for 22:6n-3 (Figure 5A, S4). Consequently, the ratios of 20:4n-6 to 22:6n-3 and the total n-6 PUFA to the total n-3 PUFA were significantly increased from passage 4 to the last passage studied (Figure 5A insert). Thus, the clearest finding in the PC and PE species profiles, the increases of 38:4 and 36:4, was supported by the observed clear accumulation of 20:4n-6 in the cellular total FA pool. Compared to the medium, in the early passage samples the relative levels (mol%-%) of 16:0 and MUFAs (16:1n-7, 18:1n-7, 18:1n-9) were lower, but those of 18:0 and PUFAs (except linoleic acid, 18:2n-6) higher (Figure 5A). In addition to the relative levels, we further studied the total amount of FAs in the cells on the culture dish and the amount of FAs available for the cells in the surrounding medium. The results demonstrated that in the medium FAs (including the essential PUFAs of the n-6 and n-3 families) were available in excessive amounts compared to the cells (Figure 5B).

Immunosuppressive capacity of hBMSCs decreases during cell expansion and correlates with the lipid changes
Using a co-culture assay, we next studied the ability of expanded hBMSCs to suppress T-cell proliferation. The early passage hBMSCs inhibited T-cell proliferation almost completely (illustrated with green line in overlay Figure 1E), and the suppression was less complete when the late passage hBMSCs were used (purple line in the overlay). To further study the correlation between immunosuppressive capacity and the identified lipid or fatty acid indicators we analysed the GPLs and FAs of hBMSCs from eight donors. These cells (from passage 4) had diverse functionality. Convincing correlation with the functionality was observed for PC species (38:4, 38:5, 34:1 and the ratio of 38:4/34:1) and fatty acids (20:4n-6, 22:6n-3 and the ratio of the two) (Table 2).

**Gene expression differences related to donor age and cell expansion**

Next, we made a gene expression analysis of the early passage (4) and late passage (8) cells. Measuring of the compositional distances for the samples from different passages by SIMCA showed that in terms of the GPL profile all the passage 4 and 6 samples were statistically significantly different from all the samples from passages 10 and higher (p-value<0.05). As the passage 8 thus represented a time point preceding changes in the metabolite profile, cells from this passage were chosen for the gene expression analysis. Altogether, 707 genes were found to be differently expressed between the old and young donor samples in passage 4 (Figure 6A). Interestingly, during expansion, the hBMSCs from the old donors showed almost 5 times more gene expression changes (576 genes) than the cells of the young donors (175 genes). Only 36 were shared between the young and old donors (Figure 6A). In addition, the variance in the fold changes was the greatest (i.e. 2.3) when comparing the old and young donors. The fold change variances due to cell expansion were 1.4 for the old and 0.7 for the young donors. Thus, cell expansion seemed to affect gene expression more in the cells of the old donors than in those of the young donors (Figure 6B).

We further investigated the enrichment of differentially expressed genes in different biological processes. In the old donors, gene ontologies (GO) relating to lipid metabolism, immunological processes as well as differentiation and developmental functions were downregulated (Figure 6C) whereas GO related to adhesion, signal transduction, and apoptosis were upregulated (Figure 6D). The expansion-induced changes (old donors) relating to cell division, aging and apoptosis were upregulated, (Figure 6E) whereas the downregulated GO terms included sterol biosynthetic processes, development and aging (Figure 6F). Results from the young donors’ group were similar but less distinct (Table S6).
As revealed by GO analysis, the expression of several genes related to lipid metabolism changed. Both ethanolamine kinase 2 and choline kinase beta had a lower expression level in the hBMSCs from the old donors than in those from the young. Scavenger receptor B 1 had lower expression and scavenger receptor B3 (also known as CD36) had higher expression level in old donors. Interestingly gene expression of phospholipid scramblase 1 was lower and putative aminophospholipid translocase (ATP10A) was higher in the hBMSCs of the old donors. Further the gene expression of phospholipid scramblase 4 was elevated during expansion. Gene expression of several other putative aminophospholipid translocases differed between young and old and further during expansion (Table S3-S5). Further inositol triphosphate receptor 1 gene expression was elevated during expansion. The gene expression of enzymes 1-acylgllycerol-3-phosphate O-acyltransferase 2 and serine palmitoyltransferase were elevated during expansion. Five genes for FA metabolizing enzymes were differently expressed (Table 3). Among them, the expression levels of three FA desaturase genes were lower in the cells of the old donors, i.e. FADS1, FADS2 and SCD, coding for fatty acid Δ5-, Δ6- and Δ9-desaturases, respectively. LysoPC acyl transferase genes LPCAT2 and LPCAT3 also had lower expression levels in the old donors. In addition, the gene producing cyclooxygenase 1 enzyme (COX1, also known as prostaglandin endoperoxide synthase PTGS1) and gene producing prostacyclin receptor (PTGIR, also known as prostaglandin I₂ receptor) were more expressed in passage 8 compared to passage 4. Finally, leukotriene C4 synthase and ELOVL fatty acid elongase 3 were expressed at lower levels in the cells of the old donors compared to those of the young.

As an indicator for the modulation of immune functions, the genes for the suppressors of cytokine signaling (SOCS1 and SOCS2) and several genes from ASB-family (Ankyrin repeat and SOCS box containing proteins) were less expressed in the old donors and interestingly ASB5 expression was increased during cell expansion (Table 3). The expression of the immunologically important COX-1 gene, PTGS1, was downregulated in the late passage cells. Interestingly, both TGFBR1 and IL6 were upregulated in the late passage cells. Other important genes related to the immune system, such as bradykinin receptor 1, HLA-DMA, HLA–DMB, HLA-DRA, CD74 and LAT2 genes, were less expressed in the early passage cells of the old donors.

During passaging in the hBMSCs of both young and old donors, the predominant gene expression changes were related to cell cycle, chromatin assembly and DNA integrity (i.e. Histone cluster genes, see Table S2). Importantly, of those 36 genes changing during passaging in both the young and old donors, 12 were histone cluster genes. (Figure 6A).
Discussion
This study focused on the membrane GPL and total FA composition of hBMSCs. The role of the donor’s age, individual variation, cell expansion and FA composition of the surrounding medium were taken into account. Since no previous studies on MSC phospholipid profiles have been reported we compared our results on the lipid profiles of hBMSCs with the previously published profiles of functionally very similar human primary fibroblasts. Our study demonstrates that at the lipid class level (Table 1), the relative amounts of PC, PS and PI of the hBMSCs were almost identical to that described for human fibroblasts. However, the relative amount of PE was much higher in the hBMSCs than reported for fibroblasts (21) or mammalian cells on average (49). The predominant PE species of the hBMSCs was 38:4 (18:0/20:4n-6) whereas in human fibroblasts PE species 36:1, 38:4, and 36:2 were present in equal amounts (21). This implies that biosynthetic or remodelling pathways of the GPLs in these two cell types have differences. This can be due to altered activities of CoA-independent transacylase (CoA-IT), which in immunologically activated cells promotes transfer of 20:4n-6 from PC to PE, from which different PLA2 readily release 20:4n-6 to be used for production of lipid mediators (50,51). In addition, the higher expression level of PE biosynthetic enzyme, ethanolamine kinase 2, found in the cells of the young donors (Table 3), may be linked to an increase in the relative amounts of PE ether lipids (Table 1).

Expansion caused further enrichment of 20:4n-6-containing PC and PE species (38:4 and 36:4), which happened at the expense of n-3 PUFA-containing species (40:5, 40:6 and 40:7) and short-chain saturated and monounsaturated species of PC and PE (Figure 4A,B). It is possible that this increase in the ratio of GPL species with n-6 PUFA to those with n-3 PUFA had consequences for the functionality of the cells, i.e. their responsiveness to inflammatory signals and capability for immunomodulation (52). If cleaved by activated phospholipases, the different PUFAs give rise to signalling molecules with different, even opposing functional consequences (53,54). This hypothesis was supported by the results obtained from the T-cell suppression assay, where we clearly showed that the cells lose part of their immunosuppressive potential when expanded (Figure 1E).

The analysis of FA composition confirmed the central feature that aging hBMSCs gained 20:4n-6 and lost 22:6n-3 and other n-3 PUFAs. The 20:4n-6 is a precursor for several pro- and also anti-inflammatory molecules such as PGs (cyclo-oxygenase COX pathway) or lipoxins (lipoxygenase LOX pathway) (29). Since the rate of COX catalysed synthesis of PGs is known to be affected by the local availability of 20:4n-6 and the expression level of the COX-1 gene (PTGS1) was elevated in the passage 8 samples, it is plausible that the
production of 20:4n-6-derived proinflammatory eicosanoids is facilitated in the aging cells (55). As described earlier by others the inducible form of cyclooxygenase, COX-2 (PTGS2) gene is highly expressed in hBMSCs (56). Our microarray data confirmed PTGS2 expression, but did not show statistically significant differences. Due to the low gene expression levels of LPCAT 2 and 3, the recycling of 20:4n-6 back to PC may have been impaired in the cells of the old donors, thus strengthening the shift to eicosanoid production. The connection to eicosanoid production is in line with the previously proposed role of eicosanoids in hMSC immunomodulative properties (7) and further supported by our observation that during expansion and simultaneous increment of 20:4n-6 content hBMSCs lose part of their capacity to suppress T-cell proliferation (Figure 1E). Similarly, we found convincing negative correlations between the immunomodulatory capacity of hBMSCs from 8 donors and the relative amounts of 20:4n-6-containing species in PC or the relative amount of 20:4n-6 in total FAs of the cells (Table 2). This encourages for further studies of the immunomodulative effector lipids affecting stem cells.

The composition of the total PUFA pool available in the cells (but not in the medium) (Figure 5 A,B) dictated the species profiles of membrane phospholipids and may also have affected the proportions of different GPL classes in the membranes, e.g. the ratio of PI/PS (Figure 3). The increase of the PI/PS ratio during expansion may be a direct consequence of the change in the cellular PUFA status due to the substrate preference in the PS biosynthesis for 22:6n-3, and in PI synthesis for 20:4n-6 (57,58).The change in PI and PS levels is biologically highly important since both of these anionic GPLs are concentrated on the inner leaflets of membranes where they activate proteins and take part in signalling events (59). Anionic lipids and PIPs as potent activators of cPLA2 release 20:4n-6 and other PUFAs (60). In addition, PI, PIPs, and PS normally function in concert to target protein kinases such as PKC, Akt, sphingosine kinase and 3-phosphoinositiode-dependent kinase-1 to membranes for activation and signalling (61-64). PIP-derived and phospholipase C-liberated DAG and PS activate the PKC, but their roles are different. Even small concentrations of DAG turn PKC on, but full enzymatic activity is achieved by PS (65). Thus, changes in the ratio of PI/PS may have profound effects on hBMSC therapeutic functions via the regulation of the activities of kinases, phospholipases and/or other proteins. We also emphasise that PIPs regulate the scavenger receptor B1 (SR-B1) of lipoprotein trafficking (66) and in our study SR-B1 was expressed at a higher level in the hBMSCs of the young donors. Interestingly, we found that gene expression of several ATPases was altered. One of the putative aminophospholipid translocases, ATP10A, and bi-directional phospholipid translocator, scramblase 1, were expressed at higher levels in the hBMSCs of the old donors. Furthermore, the gene expression
of scramblase 4 was increased during expansion (67,68) Thus our data indicates physiologically important GPL remodelling with functional consequences in the hBMSCs.

In resting cells, the amount of free PUFAs is strictly regulated by lysophospholipid acyltransferases that recycle most of the cleaved PUFAs back to the membrane GPLs. Only a small fraction of PUFAs is used for the synthesis of the eicosanoids and other signalling molecules. However, the stimulation of the cells results in the activation of cPLA_2 and due to the high rates of PUFA liberation, the shift to the production of the signalling molecules with biological activity is enforced (69). The increased concentrations of lysoPC, detected especially in the late passage hBMSC samples of the young donors, are likely to reflect enhanced PLA_2 activity (several forms of the enzyme may be activated) and represent the pool of acceptor lysophospholipids,(70,71) thus giving lysophospholipid acyltransferases a role in the regulation of inflammation (72). In this respect, the hBMSCs from the young and the old donors had different metabolism: lysoPC acyltransferases had higher expression levels and lysoPC totals also increased during expansion more noticeably in the hBMSCs of the young donors compared to the old donors (Figure S3). Additionally, lysoPC contributes to cellular signalling by activating G-protein-coupled receptors, ERK and MAPK signalling, and phospholipase C (73-75). The latter releases DAG and inositol triphosphate with the liberation of intracellular Ca^{2+} and activation of PKC. The COX enzymes reside in endoplasmic reticulum close to cPLA_2 and are likely to convert mobilized 20:4n-6 and other PUFAs into eicosanoids (54). Consequently, less free FAs may have been recycled back to PC by the lysoPC acyltransferase, which could then be observed as the lysoPC acceptor accumulation.

If the relative increase of 20:4n-6-containing GPLs of the hBMSCs have functional consequences, we can assume that the simultaneous decrease of n-3 PUFA content is of immunological importance as well. Recently, the so-called traditional eicosanoids have been found to be accompanied by resolvins, protectins and maresins, which are derivatives of 22:6n-3 and 20:5n-3 and play a role in the resolution phase of the inflammation (29). The enzymes COX and LOX synthesize from the n-3 PUFA counterparts to the n-6 PUFA-derived eicosanoids, which however have mainly anti-inflammatory properties (76). In T-cells, the n-3 PUFAs have been found to suppress immune functions by modulating interleukin-2 expression and exerting their effects via PKC isoforms and nuclear factor pathways (77). Thus, the shift from a more balanced n-6/n-3 PUFA ratio towards the dominance of n-6 PUFAs found in the total FA pool of the aging hBMSCs may have altered the lipid signalling pathways from an anti- to pro-inflammatory direction (Figure 5). Due to the constant excess amount of FAs in the surrounding medium compared to the cells (Figure 5B), we can assume
that the observed changes in hBMSC lipidome were due to an active process of the replicative senescence verified by several different markers (Figure 1A-D). However it is possible that supplementing the culture medium with even more of n-3 PUFAs than present in standard growth medium might support hBMSCs growth and maintain their physiological functionality for longer without manifestations of senescence. Indeed, it is fascinating and important to highlight that different GPL/FA compositions may cause the hBMSCs to respond very differently to the same external stimuli and that, depending on the GPL/FA substrate, the same enzymatic pathways produce different end-product eicosanoids with completely different, even opposing biological functions (55). The PUFA alterations reported here (Figure 4 and 5) presumably affect the composition of end-products involved in activation or resolution of inflammation, linked to the changes in T-cell suppression capacity (Figure 1E). Thus, our results will serve as a basis for further studies aiming to understand the lipidomic aspects of the MSC immunosuppression.

This study is the first to show major changes in phospholipid profiles and total FAs during expansion and senescence of therapeutically important hBMSCs. We demonstrate that the 20:4n-6 contents of cellular lipids increased while the n-3 PUFA contents decreased during long-term cultivation. The gene expression differences were most notable between the early passage cells from the old and the young donors and supported our lipid findings. Our results suggest that the free PUFAs derived from membrane lipids are of high importance in hBMSC immunological functionality. Since PUFA-derived signalling lipids are known to be involved in MSC functionality, but are extremely challenging to use as biomarkers, instead, biosynthetic precursors in membranes could be used as indicators of cell functionality. In combination with additional markers, such as mRNA of lipid enzymes and/or other functional factors, membrane lipids can serve as powerful new biomarkers for the functionality of therapeutic cells.
Acknowledgements
The authors would like to thank Birgitta Rantala and Lotta Sankkila for excellent technical assistance and Tanja Kaartinen for advice in T-cell proliferation assay. This work was partly supported by the SalWe Research Program for IMO (Tekes - the Finnish Funding Agency for Technology and Innovation grant 648/10) and the EVO Medical Research Fund of Finnish Red Cross Blood Service.
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chemoattractants and cationic peptides as underappreciated modulators of responsiveness to SDF-1 gradients.  
*Leukemia* **26**: 63-72.


Figure Legends

**Figure 1 Long-term cell culture of hBMSCs**
A. A representative example of cell morphology at passage 3 and in senescence (p14) at a magnification of 40x. B. hBMSCs were isolated from the human bone marrow of ten donors and cultured until senescence was reached. B. Cumulative population doublings was calculated in relation to the starting point of the cell culture at every passage (F, female; M, male). C. Western plot analysis of cell cycle components p16 and p21 of early and late passages of three different hBMSC donors. Fold change (FC) represents the increase of band intensities in late passage cells compared with early passage cells. Intensities of the p16 and p21 bands normalized to β-actin are listed on the last rows. D Telomere lengths (TRF, terminal restriction factor) were determined in early and late passage cells from three different cell lines. Results are show as mean ± standard deviation of three technical replicates. Telomere lengths were shortened on average 1 kb±0.5 (student’s t-test p-value 0.03, n=3) E. Immunosuppressive capacity of early and late passages analysed by co-culture assay. 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CSFE) –labeled peripheral blood mononuclear cells (PBMCs) were cultured with hBMSC in ratio of 1:10 for four days. T-cell proliferation was stimulated with monoclonal CD3 antibody and proliferation was analysed as CFSE dilution by flow cytometry. In the overlay panel, black line= No hBMSCs, red line=late passage hBMSCs and blue line= early passage hBMSCs. x-axis show the fluorescence intensity of CFSE on a logarithmic scale and y-axis the cell count. Experiment was repeated three times with similar results using hBMSCs and PBMCs from independent donors.

**Figure 2 Species composition in GPL classes of hBMSCs as determined by ESI-MS**
The individual lipid species were quantified using appropriate internal standards. The values for GPL species were expressed as mol% per the total of detected lipids in a given class. PC (A), PE (B), PS (C), and PI (D) species from passage 4 are shown. Results are the mean ± standard deviation (n=5) for both the young (white bars) and old (grey bars) donors (* p<0.05, ** p<0.01).

**Figure 3 The ratio of PI to PS from early to late passages of the hBMSC**
hBMSCs were cultured from the passage 4 (p4) to senescence. Cells were harvested for GPL analysis from every other passage. The GPL species were analysed after extraction by direct infusion ESI-MS. The phosphatidylinositol (PI) and phosphatidylserine (PS) class totals were obtained by summing the amounts of individual species in each class, and subsequently the ratio of PI to PS was calculated. A. Young (n=5) and B. Old donors (n=5).

**Figure 4 Effect of long-term cell culture on the PC and PE species of hBMSC.**
hBMSCs were cultured from the passage 4 (p4) to senescence. Cells were harvested for GPL analysis from every other passage. The GPL species were analysed after extraction by direct infusion ESI-MS and their values were expressed as mol% per the total of detected lipids in a given class. Results are the mean + standard deviation of hBMSCs from young (left panel) and old (right panel) donors. Paired student’s t-test was performed between the earliest and the last passages. A. PC, B. PE. C. Mean values of PC34:1, PC38:4, PE36:1 and PE38:4 in both groups over passages. D. Multivariate PCA using PC and PE data as loadings. For simplicity, the areas covering the positions of the individual sample points are shown.

**Figure 5 Effect of long-term culture on the FA composition of hBMSC**

Samples for gas chromatographic FA analysis were collected identically as described in the legend of Figure 4. FAs were analysed as methyl esters. Results for the cells from the earliest and last passages were expressed as mean+ standard deviation, n=10 (* p<0.05, ** p<0.01). For the medium 3 replicates were analysed. A. Molar percentages of FAs from the earliest (white bar) and the last passage (light grey bar) compared with those of foetal bovine serum (dark grey bar). Insert shows the ratios of 20:4n-6 to 22:6n-3 and n-6 PUFA’s to n-3 PUFA’s in the earliest and last passages compared to values of the FBSC. B. Total amounts (nmol) for each FA in the cells and surrounding culture medium. Insert shows the total amounts (nmol) of some PUFA species in enlarged scale.

**Figure 6 Differential gene expression of hBMSCs**

Differentially expressed genes between old and young donor hBMSCs in earliest passage 4 (o4-y4) and between passage 8 and 4 of old (o8-o4) and young (y8-y4) donor hBMSCs were compared. Cut-off values 0.58 (upregulated genes) and -0.58 (downregulated genes) were used. A. Venn diagram demonstrating the separate and overlapping genes in each group B. Violin plot combines boxplot with kernel density plot and is used to illustrate fold change variations and the amount of differentially expressed genes in the above-mentioned comparisons. GO enrichment analysis of differentially expressed genes is shown from C. to F. C. Genes with higher expression level in old (o4-y4) and E. genes with higher expression level in young (o4-y4). D. Genes, which expression decreased during cultivation (o8-o4) and F. genes, which expression induced during cultivation (o8-o4). The y-axis shows the GO term while the x-axis shows the enrichment-log10p-values for the enriched GO terms. For clarity, several GO terms have been excluded based on their similarity. mRNA data are available in GEO database [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39035](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39035) with GEO accession number GSE39035.
## Tables

### Table 1 Molecular percentage of lipid classes determined by ESI-MS

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Young donors</th>
<th></th>
<th>Old donors</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early passage</td>
<td>Late passage</td>
<td>Early passage</td>
<td>Late passage</td>
</tr>
<tr>
<td>PC diacyl</td>
<td>37.3±2.7</td>
<td>37.4±3.4</td>
<td>35.5±2.4</td>
<td>39.0±3.7</td>
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<tr>
<td>PC alkyl</td>
<td>5.8±0.3</td>
<td>5.1±0.4**</td>
<td>5.3±0.5</td>
<td>6.6±0.8*</td>
</tr>
<tr>
<td>PE diacyl</td>
<td>20.4±1.1</td>
<td>15.1±2.1*</td>
<td>19.9±3.8</td>
<td>17.2±3.4</td>
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<tr>
<td>PE alkenyl</td>
<td>16.1±2.1</td>
<td>22.7±4.7*</td>
<td>16.8±2.6</td>
<td>16.5±1.9</td>
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<tr>
<td>PI</td>
<td>3.9±1.2</td>
<td>7.7±1.7*</td>
<td>5.4±1.3</td>
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<tr>
<td>PS</td>
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<td>4.9±1.1</td>
<td>5.3±1.1</td>
<td>5.9±2.1</td>
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<td>PI/PS</td>
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<td>lysoPC</td>
<td>0.5±0.1</td>
<td>1.5±0.9</td>
<td>1.2±0.5</td>
<td>1.2±0.6</td>
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</table>

The totals for lipid classes were calculated by summing up individual lipid species of each class. The statistical difference between the young and the old donors was calculated using student’s two-tailed t-test (paired). Abbreviations: lysoPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, Phosphatidylserine;
Table 2. Regression analysis of correlation between immunosuppression capacity and the best lipid indicators in hBMSCs.

<table>
<thead>
<tr>
<th>lipid or fatty acid indicator</th>
<th>correlation coefficient</th>
<th>R squared</th>
<th>p-level</th>
<th>value range (mol%)</th>
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<tr>
<td>PC38:4(chains 18:0, 20:4n-6)</td>
<td>−0.91</td>
<td>0.84</td>
<td>0.001</td>
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<td>PC38:5(chains 18:1, 20:4n-6)</td>
<td>−0.82</td>
<td>0.68</td>
<td>0.012</td>
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<td>PC34:1(chains 16:0, 18:1)</td>
<td>+0.80</td>
<td>0.64</td>
<td>0.017</td>
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<td>PC38:4/PC34:1</td>
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<td>0.81</td>
<td>0.003</td>
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<tr>
<td>20:4n-6</td>
<td>−0.71</td>
<td>0.50</td>
<td>0.050</td>
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<tr>
<td>22:6n-3</td>
<td>+0.75</td>
<td>0.56</td>
<td>0.032</td>
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<td>20:4n-6/22:6n-3</td>
<td>−0.76</td>
<td>0.58</td>
<td>0.029</td>
<td>2.2-4.9</td>
</tr>
</tbody>
</table>

The hBMSCs (n=8, all cultured till passage 4) suppressed T- cell proliferation in a co-culture assay (degree of inhibition with hBMSC:PBMC ratio 1:20; range 70-88%) and relative amounts of selected lipid and fatty acid indicators or their ratios in the cells.
### Table 3 Differentially expressed genes

<table>
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<tr>
<th>Description</th>
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<th>Gene ID</th>
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<td>Lipid metabolism</td>
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<td>1-acylglycerol-3-phosphate O-acyltransferase 2</td>
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<td>CD36 molecule (thrombospondin receptor)</td>
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<tr>
<td>ELOVL fatty acid elongase 3</td>
<td>ELOVL3</td>
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<td>Ethanolamine kinase 2</td>
<td>ETNK2</td>
<td>55224</td>
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<td>Fatty acid desaturase 1(delta -5-desaturase)</td>
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<td>Fat storage-inducing transmembrane protein 2</td>
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<td>LAT2</td>
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</table>

Differentially expressed genes related to lipid metabolism and immune response. 04-y4 comparison between old and young donor’s samples in passage 4, o8-o4 comparison between old donors samples in passage 8 and 4.
Old Donors

Young Donors