

Restored *in vivo*-like membrane lipidomics positively influence *in vitro* features of cultured mesenchymal stromal/stem cells derived from human placenta

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Abstract

Background: The study of lipid metabolism in stem cell physiology has recently raised great interest. The role of lipids goes beyond the mere structural involvement in assembling extra- and intra-cellular compartments. Nevertheless, we are still far from understanding the impact of membrane lipidomics in stemness maintenance and differentiation patterns. In the last years, it has been reported how *in vitro* cell culturing can modify membrane lipidomics. The aim of the present work was to study the membrane fatty acid profile of Mesenchymal Stromal Cells (MSCs) derived from human Fetal Membranes (hFM-MSCs) and to correlate this to specific biological properties by using chemically-defined tailored lipid supplements (Refeed[®]).

Methods: Freshly isolated hFM-MSCs were characterized for their membrane fatty acid composition. hFM-MSCs were cultivated *in vitro* following a classical protocol and their membrane fatty acid profile at different passages was compared to their *in vivo* one. A tailored Refeed[®] lipid supplement was developed with the aim of reducing the differences created by the *in vitro* cultivation and was tested on cultured hFM-MSCs. Cell morphology, viability, proliferation, angiogenic differentiation and immunomodulatory properties after *in vitro* exposure to the tailored Refeed[®] lipid supplement were investigated.

Results: A significant modification of hFM-MSC membrane fatty acid composition occurred during *in vitro* culture. Using a tailored lipid supplement, the fatty acid composition of cultured cells remained more similar to their *in vivo* counterparts, being characterized by a higher polyunsaturated and omega-6 fatty acid content. These changes in membrane composition had no effect on cell morphology and viability, but were linked with increased cell proliferation rate, angiogenic differentiation and immunomodulatory properties. In particular, Refeed[®]-supplemented hFM-MSCs showed greater ability to express fully functional cell membrane molecules.

Conclusions: Culturing hFM-MSCs alters their fatty acid composition. A tailored lipid supplement is able to improve *in vitro* hFM-MSC functional properties by recreating a membrane environment more similar to the physiological counterpart. This approach should be considered in cell therapy applications in order to maintain a higher cell quality during *in vitro* passaging and to influence the outcome of cell-based therapeutic approaches when cells are administered to patients.

Background

In regenerative medicine, high therapeutic promises have been based on the possibility of stimulating *ex vivo* and *in vitro* expansion of stem cells and their differentiation into functional progeny that could regenerate injured tissues/organs in humans [1, 2]. Alterations in cell properties may occur during *in vitro* manipulation due to biochemical or biophysical changes from *in vivo* physiological conditions to *in vitro* ones [3, 4]. Without proper culture systems and protocols, stem cells cannot grow normally outside the body and will gradually lose their multipotency or pluripotency (i.e. they will differentiate), and can undergo early senescence [5, 6]. Substrate surface chemistry and culture medium composition are the two main factors that researchers have spent a long time trying to optimize for *in vitro* cell culture [7]. The development of a fully defined and xeno-free system (i.e. chemically and physically defined) is required in stem/somatic cell culture to provide a “non-contaminated” cell population for cell therapy and tissue regeneration to eliminate safety concerns related to clinical use.

In this context, lipid metabolism is pivotal in stem cell physiology and it plays a central role in stem cell maintenance and differentiation [8-10]. However, the full understanding of stem cell lipid metabolism is still far away, but once achieved it could bring great advances in their handling and use. Kang and co-workers recently reviewed the preliminary efforts produced by the stem cell community in investigating the *in vitro* regulation of stem cell proliferation and differentiation by essential fatty acids and their metabolites [11].

Among stem cell populations, Mesenchymal Stromal Cells (MSCs) draw the attention of the scientific community for their widespread properties, functions

and clinical applications [12, 13]. One of the most promising functions revolves in their interaction with the immune system, modifying its response [14, 15]. MSCs can be isolated from several fetal, perinatal and adult tissues [16]. Placenta-derived tissues (i.e. placenta, fetal membrane and umbilical cord) are interesting sources of MSCs for research purposes and clinical applications due to their readily availability and easy recovery without any ethical concerns [17]. These perinatal cells are able to proliferate *in vitro* for several passages [18] and present the classical MSC immunophenotype (CD44⁺, CD90⁺, CD73⁺, CD14⁻, CD 31⁻, CD34⁻, CD45⁻) [19]. They can differentiate through the three classical mesodermal lineages (osteogenic, chondrogenic and adipogenic) [20-22] and also into endothelial [23], hepatocytic [24] and myogenic [25] lineages, but present differences depending on the origin of the cells. Moreover, Placenta-Derived Stromal Cells have been demonstrated to preserve their plasticity alongside the maintenance of fetal tolerance due to their immunomodulatory properties [26-28].

These two biological characteristics could be useful for promising cell therapy approaches in regenerative medicine. Moreover, mesenchymal cells isolated from human placenta have been demonstrated to treat ischemia in an animal model [29] and reduce inflammation in pre-eclampsia-like features during pregnancy in an animal model [30]. The *in vivo* characteristics and properties of MSCs should be preserved *in vitro*, in order to study and more effectively utilise MSCs.

Similar to the other typologies of stem cells, MSCs show a clear preference for ATP production through glycolysis, rather than by using glucose and fatty

acid oxidation [31]. To date, only a few studies on MSC membrane lipids have been performed [32, 33], and membrane lipid composition during *in vitro* expansion of MSCs has not been studied systematically. Changes in the membrane fatty acid profile during MSC differentiation [34-36] and results obtained by lipid supplementation [37] show how membrane lipids play a crucial role in MSC metabolism, even if this is still mainly unexplored.

As shown elsewhere [38], an adequate integrative lipid supplementation is fundamental to improve the quality of *in vitro* cells and to modulate specific cellular properties of interest. In fact, in the majority of cases, cultured cells develop a membrane network that is altered and non-functional to their physiology, thus reducing their full potential.

In this work, we investigated how the membrane fatty acid composition of MSCs derived from human Fetal Membranes (hFM-MSCs) is modified by the *in vitro* culturing process. We showed that a tailored lipid supplement is able to maintain *in vitro* a membrane lipid pattern more similar to the physiological one. Finally, we explored how membrane lipid changes are able to impact on many relevant biological properties of *in vitro* hFM-MSCs and hereby we discuss potential implications of lipid monitoring and supplementation in cell therapy applications and related therapeutic approaches.

Materials and Methods

Isolation and cultivation of fetal membrane mesenchymal stromal cells

Term placentas were obtained by caesarean section from healthy donor mothers, after written informed consent and according to the policy of the Local Ethical Committee. The study was approved by Local Ethical

Committee: St. Orsola-Malpighi University Hospital Ethical Committee, protocol number 1645/2014, ref. 35/2014/U/Tess).

Fetal membrane samples were separated from the chorionic plate and washed in phosphate buffered saline (PBS, Lonza, Walkersville, MD, USA) containing 1% penicillin-streptomycin solution (Lonza, Walkersville, MD, USA). For hFM-MSC isolation, small fragments of membranes were minced and then digested using a solution of 1 mg/ml collagenase type IV (Sigma-Aldrich Co., St. Louis, MO, USA) and 0.25% trypsin-EDTA (Lonza, Walkersville, MD, USA). The fragments were resuspended for 15 min, and then incubated for 15 min at 37°C twice. Fetal bovine serum (FBS, Lonza, Walkersville, MD, USA) was added and a final centrifugation at 400 g for 10 min was performed. The pellet was resuspended and cells were plated in culture flasks in DMEM with high glucose (Lonza, Walkersville, MD, USA) and supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin solution.

Membrane isolation following culture

Cells (7×10^6) were collected in a 15 ml tube, centrifuged at 500 x g for 5 min and resuspended in 10 ml of PBS. The wash was repeated five times in order to remove traces of medium and serum used during the culture process. Cells were then resuspended into 500 μ l of PBS and collected in a 1 ml tube, to which 500 μ l of sterile H₂O were added. Cells were then centrifuged for 30 min at 15,000 x g in a refrigerated centrifuge at 4°C. The collected membranes were resuspended in 1 ml of PBS: H₂O 1:1 and washed 5 times following the same procedure.

Fatty acid composition analysis

Cell membrane lipids were extracted with $\text{CHCl}_3/\text{MeOH}$ 2:1 (vol/vol) and then incubated with 0.5 M KOH in methanol for 10 min at room temperature, thus trans-esterifying fatty acids linked by ester bonds to methanol so forming Fatty Acid Methyl Esters (FAMEs). FAMEs were extracted with n-hexane and separated by gas chromatography in an Agilent 7820A GC System (Agilent Technologies, Santa Clara, USA) fitted with a 60 m x 0.32 mm DB23 capillary column, film thickness 0.25 μm , and a Flame Ionization Detector (FID). Helium was used as carrier gas at 2.54 ml/min and the split injector was used with a split ratio of 10:1. Injector temperature was 250°C and detector temperature was 260°C. The column oven temperature was maintained at 50°C for 2 min after sample injection and was programmed for the following temperature gradient: 10°C/min from 50°C to 180°C, 3°C/min from 180°C to 200°C and holding at 200°C for 6 min. The separation was recorded with G6714AA SW EZChrom Elite Compact (Agilent Technologies). FAMEs were identified by comparison with standards purchased from NuCheckPrep Inc., Elysian, USA. FAMEs are expressed in weight %, based upon the % contribution of the peak area of each FAME in the chromatogram. To take into account the different signal of the detector for different molecules, a correction factor was applied to the experimental data coming from the integration of the chromatograms. The total of the peaks analysed for each chromatographic run was 100.

Fatty acid aggregates were calculated as follows:

$$\Sigma \text{ Saturated Fatty Acids (SFAs)} = 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 +$$

22:0 + 23:0 + 24:0;

Σ Monounsaturated Fatty Acids (MUFAs) = 16:1n-7 + 18:1n-9 + 18:1n-7 +

20:1n-9 + 22:1n-9 + 24:1n-9;

Σ Polyunsaturated Fatty Acids (PUFAs) = 18:2n-6 + 18:3n-6 + 18:3n-3 +

20:3n-9 + 20:3n-6 + 20:4n-6 + 20:3n-3 + 20:5n-3 + 22:2n-6 + 22:4n-6 + 22:5n-6 + 22:5n-3 + 22:6n-3;

Σ Omega-3 = 18:3n-3 + 20:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3;

Σ Omega-6 = 18:2n-6 + 18:3n-6 + 20:3n-6 + 20:4n-6 + 22:2n-6 + 22:4n-6 + 22:5n-6.

Refeed[®] supplements

Refeed[®] supplements (Remembrance Srl, Imola, Italy) are a completely defined combination of non-animal derived lipids and antioxidants (NuCheckPrep Inc., Elysian, USA; Sigma Aldrich Co., St. Louis, MO, USA; Applichem an ITW Inc., Chicago, USA) solubilised in 1 ml of ethanol (Sigma-Aldrich Co., St. Louis, MO, USA). 1 ml of Refeed[®] was diluted in 500 ml of complete cell growth medium, the resulting ethanol concentration being < 1% (vol/vol) in the final medium. Refeed[®] composition is shown in Table 1. Starting from the first passage (P1), treated cells were supplemented with the full dose of Refeed[®]. During passage 0 (P0) the full dose was divided into three equal doses and supplemented at different times, in order to allow cells to adapt to the supplement.

Table 1. Composition of Refeed[®] used for *in-vitro* supplementation of hFM-MSCs.

	Refeed [®]
Lipids	2.14
Antioxidants	.62

Data are the amount (mg) per 500 ml of complete medium.

Immunofluorescence

Cells were seeded (2.5×10^4) onto glass coverslips and fixed for 10 min in 2% Paraformaldehyde at RT. After three washes in PBS, cells were permeabilized for 10 min with PBS 1% Triton (Triton X-100, Sigma-Aldrich, Co., St. Louis, MO, USA), then incubated in blocking solution 1X PBS 1% BSA (Sigma-Aldrich, Co., St. Louis, MO, USA) for 30 min. Primary antibody anti-Vinculin (1:100, Chemicon, Temecula, CA, USA) diluted in blocking solution was added and incubated for 1 h at RT. After three washes in 1X PBS, secondary antibody anti-mouse Cy3 (1:100, Sigma-Aldrich, Co., St. Louis, MO, USA) was added diluted in blocking solution and incubated for 45 min at RT. Cortical actin was stained using FITC-Phalloidin (1:250, Sigma-Aldrich, Co., St. Louis, MO, USA) and added directly during secondary antibody incubation. Coverslips were mounted after washes in PBS in ProLong Gold Antifade Mountant with Dapi (Thermo Fisher Scientific, Monza, MB, Italy)

Cell proliferation

hFM-MSCs from passage 1 to 8 were analysed for their population doubling, population doubling time and cumulative population doubling. Cells were seeded at an initial concentration of 5000 cells/cm² in T25 culture flasks. At every passage, cells were harvested with 0.25% trypsin-EDTA solution for 3 min at 37°C and counted with a haemocytometer by erythrosine B exclusion.

Flow cytometry characterization

hFM-MSCs at the third passage were analyzed by flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher Scientific) to verify their immunophenotypic profile. The antibodies used were for hematopoietic markers (anti-CD14-allophycocyanin (APC), anti-CD34-fluorescein isothiocyanate (FITC) (Becton Dickinson) and anti-CD45-Peridinin Chlorophyll Protein complex PerCP) (Becton Dickinson)); for an endothelial-perivascular marker (anti-CD31-phycoerythrin (PE) (Becton Dickinson)); for stromal markers (anti-CD44-FITC (Biolegend), anti-CD73-PE (Becton Dickinson), anti-CD90-PE (Biolegend)); and for the major histocompatibility complex class II (anti-HLA-DR-PE (Becton Dickinson)).

Angiogenic differentiation

In order to induce cells through the angiogenic lineage they were cultured in with and without Refeed[®] in DMEM containing 2% FBS with the addition of 50 ng/ml of Vascular Endothelial Growth Factor (VEGF, Sigma-Aldrich Co., St. Louis, MO, USA) for 6 days, changing the medium every 2 days. As a

negative control of angiogenesis, cells with and without Refeed[®] were cultured in standard medium, DMEM with 10% FBS. Then cells were fixed for flow cytometric analysis and the expression of FLT1 (VEGFR1, R&D system), KDR (VEGFR2) and Von Willebrand Factor (vWF, Abcam) was measured.

Immunomodulation

In order to investigate immunomodulatory properties of hFM-MSCs on activated peripheral blood mononuclear cells (PBMCs), they were plated in 6-well plates at a density of 10,000 cells/cm² and allowed to stabilize in culture for 1 day. PBMCs were isolated from the blood of healthy donors by density gradient centrifugation (Ficoll-Paque, Sigma-Aldrich) and co-cultured on hFM-MSC monolayers at a ratio of 10:1 in RPMI with 10% FBS (Lonza, Walkersville, MD, USA). PBMCs were activated by addition of phytohemagglutinin (PHA, 1 µg/ml, Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for 72 h at 37°C, 5% CO₂. The negative control was PBMCs without PHA stimulation and the positive control consisted of PBMCs stimulated by PHA in the absence of hFM-MSCs. The immunomodulatory ability of hFM-MSCs was quantified by BrdU incorporation by activated PBMCs. After 72 h of co-culture between hFM-MSCs and PBMCs, the latter were recovered and seeded in a 96-well plate and then BrdU incorporation levels were quantified using a colorimetric immunoassay, according to the manufacturer's instructions (Cell Proliferation ELISA, BrdU – colorimetric kit, Roche, Basel, Switzerland). hFM-MSCs cultured with and without activated PBMCs were fixed for flow cytometric analysis. Finally, HLA-G (Abcam

ab7904) expression of hFM-MSCs was measured to confirm the immunomodulatory phenotype.

Statistical analysis

All the experiments were performed at least three times. Data are presented as means \pm standard deviation and were analysed by one and two-way Anova or t test using Graph Pad Prism software. The significance threshold was $p < 0.05$.

Results

Cultured hFM-MSCs have significantly different membrane fatty acid composition compared to their fresh uncultured counterparts

Fresh uncultured hFM-MSCs showed variability in their membrane fatty acid composition, likely due to the donors' genetic and lifestyle diversity. They had a membrane fatty acid composition mainly characterised by a high omega-6 fatty acid content, which represented more than 25% of total membrane fatty acids. The effect of culturing human hFM-MSCs in the traditional medium (DMEM + 10% FBS) on their membrane fatty acid composition was investigated and results are shown in Figure 1 (black squares). Data clearly demonstrate that significant differences arise during the first *in vitro* passages (mainly P0 and P1) between the membrane fatty acid profiles of *in vitro* cultivated hFM-MSCs and those of the corresponding fresh uncultured hFM-MSCs, considering both fatty acid sums (Figure 1 black squares) and individual fatty acids (data not shown). Overall, cultured cells had lower proportions of PUFAs than freshly isolated cells. In particular, they showed a

large drop in omega-6 fatty acids, counterbalanced by a marked increase in MUFA and omega-3 fatty acids (Figure 1 black squares).

Refeed[®] supplementation partially realigns hFM-MSC membrane fatty acid composition to that of their fresh uncultured counterparts

hFM-MSCs were cultured in the traditional medium (DMEM + 10% FBS) supplemented with specific Refeed[®] supplements, which are completely defined combinations of lipids and lipophilic antioxidants in ethanol (see Materials and Methods). Ethanol and antioxidants did not show any effect on cultured hFM-MSCs when tested as a negative control (data not shown). Culture with a tailored Refeed[®] formulation was able to partly prevent the changes induced by the traditional *in vitro* culture system and to restore over time the membrane fatty acid profile to one that better matched that of fresh uncultured hFM-MSCs (Figure 1, white squares). In particular, Refeed[®] supplementation was able to partly reduce the loss of PUFA and of omega-6 fatty acids in particular, while decreasing the accumulation of MUFA and omega-3 fatty acids. Individual fatty acids followed the same fluctuations (data not shown). Therefore, the membrane network of Refeed[®] supplemented hFM-MSCs better mimics that of fresh uncultured hFM-MSCs in its fatty acid composition and so most likely in its biophysical and functional properties.

Isolation and proliferation

In order to evaluate the effect of Refeed[®] on cultured hFM-MSCs, cells were isolated and cultured *in vitro* with and without supplementation until passage eight (P8). Cells cultured with Refeed[®] showed a morphology similar to

control cells, without lipid accumulation despite supplementation (Figure 2 a-b). In order to investigate also the cytoskeleton structure and the cell adhesion, in particular the focal adhesion complexes, an immunofluorescence for phalloidin and vinculin was performed. Cells cultured with Refeed[®] showed no changes to the cytoskeleton structure nor to the adhesion complex distribution compared to control cells (Figure 2 c-d). At each passage cells were counted and it has been calculated population doubling, population doubling time and cumulative population doubling. Figure 3 represents the theoretical number of cells obtained from initial cell seeding, valuated at cumulative population doubling obtained for each passage from 1 to 8. The increase in cell number, reflecting the rate of proliferation, was greater for cells cultured with Refeed[®] (Figure 3).

Immunophenotypic analysis

Cell surface antigens were assessed by flow cytometry analysis for a variety of markers associated with hematopoietic (CD14, CD34 and CD45), mesenchymal stromal (CD44, CD73 and CD90), and mature endothelial (CD31) cells. Finally, the major histocompatibility complex class II HLA-DR was also analysed. Figure 4 shows that cells expanded for three passages in culture with Refeed[®] supplementation exhibited a similar immunophenotype to cells cultured in normal conditions, with high expression of stromal markers, and low expression of hematopoietic, endothelial markers and HLA-DR.

Angiogenic differentiation

In order to understand the biological and functional effect of Refeed[®] we have studied in detail angiogenic differentiation. Cells were induced for six days with VEGF and then fixed and analysed by a flow cytometry procedure for the expression of FLT1, KDR and vWF. As shown in Figure 5, there was a clear increase of both VEGF receptors (FLT1 and KDR) and of the typical endothelial cell marker vWF expression in Refeed[®] supplemented cells after angiogenic stimulus.

Immunomodulation

Another important aspect of MSCs when clinical application is concerned is their ability to suppress the immune system. A co-culture of hFM-MSCs and PHA-activated PBMCs was performed and after three days BrdU incorporation and PBMC cell cycle were analysed. Figure 6a show how Refeed[®] supplementation increased the ability of hFM-MSCs to inhibit PBMC proliferation *in vitro*. In particular activated PBMCs show a significantly lower BrdU incorporation when co-cultured with hFM-MSCs maintained in culture medium supplemented with Refeed[®] (43.27 ± 11.55 %) compared to when they were cultured in standard conditions (75.25 ± 7.02 %). These results suggest that hFM-MSCs have an immunomodulatory effect on stimulated PBMCs mediated by lymphoproliferative inhibition and this effect is increased when cells were supplemented with Refeed[®].

To support these data, we also analysed the effect of Refeed[®] supplementation on the expression of HLA-G, one of the possible mediators of the effect previously described. In cells cultured in standard medium, HLA-G expression increase from $34,6 \pm 4.1$ to 55.9 ± 2.6 % after co-culture with

PHA-activated PBMCs whereas in FM-MSCs supplemented with Refeed[®] it increased from 46.2% ± 15.6 to 75.9 ± 10.7 % after co-cultured with PHA-activated PBMCs. Figure 6b shows that HLA-G expression on hFM-MSCs increases in co-culture with PBMCs and that the increase is greater in Refeed[®]-supplemented hFM-MSCs.

Discussion

Membrane lipidomics is emerging as a core research field in stem cell biology [8-10]. Membrane lipids, including the fatty acid constituents, regulate many important aspects of stem cell physiology, stemness and differentiation [11], although we are a long way from understanding the complete picture. *In vitro* culture conditions are considered important to generating safe and effective stem cells for clinical use in humans [5, 6]. However, current *in vitro* conditions for the culture of stem cells are still far from being optimized and much more needs to be done to achieve a fully efficient *in vitro* cultivation processes, in order for stem cells to be used in large-scale industrial processes of cell therapy and regenerative medicine applications [5, 6]. In this context, membrane lipidomics has not yet been considered as a key factor and judicious lipid supplementation of *in vitro* stem cell cultures is limited and poorly thought out. This most likely reduces the potential of stem cell based therapies.

Providing fatty acids to cultured cells is known to alter the fatty acid composition of their cell membrane and this is associated with changes in cell function [37, 38]. However, such effects are poorly studied and defined in stem cells. To investigate the impact that tailored lipid supplements, and thus

different membrane lipidomics, could have on stem cells, we have chosen to study hFM-MSCs, perinatal cells derived from placental tissues, that are widely available, easy to recover and raise no ethical issues [17]. These cells show stem-cell-like features, such as high stemness and wide differentiation potential [20-25]. Moreover, these cells can be cultured *in vitro* for many passages and be expanded in sufficient number for clinical applications [18].

We showed that culture of hFM-MSCs resulted in marked changes in their fatty acid composition away from that seen in freshly isolated cells. A striking decrease of omega-6 fatty acids affected hFM-MSC membrane composition during the first *in vitro* passages (Figure 1), probably due to the inability of hFM-MSCs to generate omega-6 fatty acids internally and to the shortage of omega-6 fatty acids provided by serum. With the decrease in membrane fluidity caused by the drop of omega-6 fatty acid double bonds, cells increased MUFA synthesis through the upregulation of the Δ^9 -desaturase activity, thus lowering SFAs and raising MUFA content in the membrane system (Figure 1). MUFAs partially counterbalanced the decrease in membrane fluidity, but not in membrane plasticity, as they are not able to generate bioactive lipids like omega-6 can do [39].

Further, we identified that a tailored lipid supplementation allows hFM-MSCs to maintain a fatty acid composition that more closely resembles that of freshly isolated cells, so preventing membrane changes induced by *in vitro* cultivation (Figure 1). This was associated with improved biological characteristics of cultured hFM-MSCs. For example the observed increase in hFM-MSC proliferation, without any morphology or immunophenotype alterations, indicates an overall improvement in cell welfare, probably due to a

membrane network with a lipid composition more similar to that of the corresponding *in vivo* cells and to a better metabolism (Figure 2, 3, 4).

Increased angiogenic differentiation, documented by higher expression of a distinctive marker of endothelial cells (von Willebrand Factor) (Figure 5), is a sign of a greater cell plasticity, which suggests the likelihood of a greater efficiency in other differentiation processes. This is consistent with the higher presence of the two main receptors involved in angiogenesis (FLT-1 and KDR) in the plasma membrane of cells treated with Refeed[®] (Figure 5). This suggests that the biological stimulus, in this case the differentiation stimulus, is more efficiently supported when the cells have a fatty acid composition more like the one they had *in vivo*. In addition to the higher number of these receptors in the membrane, it is likely that also the signalling process shows greater efficiency, due to an increase in membrane domain stability provided by a different membrane order [40, 41].

The improvement of immunomodulatory capacity of hFM-MSCs seen after culture in the presence of Refeed[®] (Figure 6), albeit small, suggests an improvement also of the process of protein synthesis, protein folding and trafficking, through which the receptors involved are transferred to the plasma membrane. In addition, a greater presence of bioactive lipids derived from omega-6 fatty acids, such as lipoxin A₄, could be important in assuring a more optimal functioning of hFM-MSCs after culture in the presence of Refeed[®] [42].

Finally, experiments not reported here indicate a greater resistance of hFM-MSCs to freeze/thaw processes (data not shown) and a marked improvement

in the isolation efficiency of post enzymatic digestion of hFM-MSCs treated with Refeed[®] (data not shown).

The effects described above originate from specific *ad-hoc* lipids that are used by the cell for the creation of a membrane network with different and more efficient features than seen if those lipids are not provided to the cells. A greater presence of PUFAs, particularly of omega-6 fatty acids, makes the membrane more fluid and more plastic [39], respectively due to the presence of a greater number of double bonds (compared with SFAs and MUFAs) and a greater presence of bioactive lipids. Greater efficiency in the process of trafficking, transmembrane protein folding and signalling pathways could contribute to improve the global efficiency of the whole cell. Another interesting hypothesis comes from recent work [43] showing that an increase in membrane fluidity positively affects the solubility and diffusion of oxygen in membranes, ensuring a greater supply to mitochondria. This would improve oxidative metabolism.

From the results presented herein, culture of hFM-MSCs in the presence of the Refeed[®] lipid supplement creates *in vitro* conditions more similar to the *in vivo* one and contributes to a more efficient cell physiology and metabolism, thus enabling the maintenance of many cellular responses and functions. It will be important to conduct a thorough study of the molecular changes that occur during the culture of these cells and how these changes are influenced by Refeed[®]. Among the most intriguing future perspectives, there is the possibility to investigate if and how the paracrine activity of these cells could possibly be influenced by an improved membrane lipid composition and influence in turn the same or other cell types.

Conclusions

It is now established that membrane lipidomics plays a fundamental role in cell physiology of stem cells and of MSCs in particular, although much still remains to be discovered. In order to achieve an intensive use of these cells in cell therapy and regenerative medicine applications, it is necessary to make *in vitro* cultivation processes more efficient for them to be more suitable for their large-scale industrial use. It is in this context that membrane lipidomics and related tailored membrane lipid supplements can bring important advantages in the improvement of both *in vitro* amplification processes and the outcome of administering the same to patients, through an improvement in the quality of the cell and a realignment to *in vivo* conditions that enable the expression of the cells full potential. In this regard culture of hFM-MSCs in the presence of the Refeed[®] lipid supplement creates *in vitro* conditions more similar to the *in vivo* one and contributes to a more efficient cell physiology and metabolism, thus enabling the maintenance of many cellular responses and functions.

Figure legends

Figure 1. Partially preserved cultured hFM-MSC membrane fatty acid profile after Refeed[®] lipid supplementation.

The main fatty acid parameters characterizing the membrane fatty acid profile of fresh uncultured hFM-MSCs (ISO), and how they are modified during normal and Refeed[®]-supplemented *in-vitro* culturing at different passages (p0,

p1, p2, p4, p6, p8). Data are expressed in weight % of total membrane fatty acids and presented as means \pm SD (n=8 for ISO, n=5 for *in vitro* cultivated hFM-MSCs).

Figure 2. Unchanged hFM-MSC morphology after Refeed[®] lipid supplementation.

Light microscopy images of expanded hFM-MSCs cultured in traditional medium (a) and with Refeed[®] supplementation (b). Immunofluorescence images for cytoskeleton marker (phalloidin - green) and focal adhesion complexes (vinculin - red) of hFM-MSCs cultured in traditional medium (c) and with Refeed[®] supplementation (d). Nuclei were stained with Dapi (blue).

Figure 3. Increased hFM-MSC growth kinetics after Refeed[®] lipid supplementation.

Theoretical *in vitro* expansion of hFM-MSCs. The number of cells was obtained considering the initial cell seeding (5000 cells/cm²) and the related cumulative population doubling. Control cells are presented as black squares and cells supplemented with Refeed[®] as white squares.

Figure 4. Unchanged hFM-MSC immunophenotype after Refeed[®] lipid supplementation.

Representative flow cytometry analysis of mesenchymal (CD44, CD73, CD90), hematopoietic (CD14, CD34, CD45), endothelial (CD31) markers and major histocompatibility complex class II (HLA-DR). Unstained controls are

presented as filled black histograms, the specific cell markers as grey histograms.

Figure 5. Improved hFM-MSC angiogenic differentiation after Refeed[®] lipid supplementation.

Cells were induced with VEGF without (Ctrl) and with Refeed[®] supplementation. Representative flow cytometry analysis of VEGF receptors, FLT1 and KDR, and Von Willebrand Factor. Control cells are presented as filled black histograms and induced cells as white histograms.

Figure 6. hFM-MSC enhanced immunosuppressive effect on activated peripheral blood mononuclear cells after Refeed[®] lipid supplementation.

(a) Analysis of the BrdU incorporation into PBMCs after co-culture with hFM-MSCs with and without Refeed[®] supplementation. Data are expressed as percentage of BrdU incorporation and presented as means \pm SD (n=3, ***P<0.05). (b) Representative flow cytometry analysis of HLA-G expression on hFM-MSCs. Control cells are presented as filled black histograms and co-cultured cells as white histograms.

List of Abbreviations

hFM-MSCs: human Fetal Membrane Mesenchymal Stromal Cells from human fetal membrane

PBMCs: Peripheral Blood Mononuclear Cells

VEGF: Vascular Endothelial Growth Factor

vWF: Von Willebrand Factor

FAME: Fatty Acid Methyl Esters

MUFA: Monounsaturated Fatty Acids

PUFA: Polyunsaturated Fatty Acids

SFA: Saturated Fatty Acids

Declarations

Ethics approval and consent to participate

The study was approved by Local Ethical Committee: St. Orsola-Malpighi University Hospital Ethical Committee, protocol number 1645/2014, ref. 35/2014/U/Tess).

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during and/or analysed during the current study are not publicly available due to the know-how management policy of Remembrane srl but are available from the corresponding author on reasonable request.

Competing interests

AC and PP are co-founders and shareholders of Remembrane Srl, a manufacturer of lipid supplements for use in in-vitro culturing.

MR, FA, CZ, CM, FR, PLT, VT, PCC, LB declare that they have no competing interests.

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Authors' contributions

AC, PP, MR and FA conceived and designed the study; MR, CZ, CM, VT and FR performed the experiments; PP performed gas chromatography analyses; AC, MR, FA and PLT analyzed and interpreted the data; AC, MR, PCC and LB prepared the manuscript. All authors have read and approved the final manuscript.

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