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Label-free live characterization of mesenchymal stem cell spheroids by biophysical properties measurement

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ABSTRACT

Three-dimensional (3D) cell culture has become a consolidated method in the stem cell field, where mesenchymal stromal stem cells (MSCs) can be used to generate *in vitro* spheroid aggregates called MSC-Spheroids (MSph). MSph is a floating cluster of stem cells similar to those in literature are known as bone marrow-derived "mesenspheres". Even though MSC properties are shared by MSph, depending on the cell type and their tissue source, the morphology and degree of compaction of the MSph can be variable, creating limitations in such a cell model. Thus, during culture, a variation in stem cell functionality and viability, in addition to the suitability for comparing MSph in some experimental protocols, can be affected by spheroid biophysical intrinsic properties like mass density. To investigate this limitation and provide a new method for researchers, MSph of seven different tissue sources were compared by combining mass density, weight, and size evaluations with viability assays for ATP measurement. MSph cultured in traditional static conditions showed decreased in viability over the days of culture, while mass density exhibited different trends among cell types. Additionally, treatment of MSph with a non-toxic concentration of a natural compound cell regulator, such as plumbagin, altered the mass density of a selected cell type, thereby confirming the efficacy of the biophysical approach in monitoring MSph variability post-treatment. The results encourage using MSph in the early days of culture after their formation to ensure viability and likely retention of the stem cell phenotype.

1. Introduction

3D scaffold-free culture of stem cells is increasingly demanded for translational applications in clinical for regenerative medicine. Mesenchymal stromal stem cells (MSCs) easily form spheroids *in vitro* (MSph), which are believed to enhance functional properties by mimicking the native *in vivo* stem cell environment. Asking for maintening this original condition, many protocols has allowed the easy generation of spheroids *in vitro* called mesenspheres.

The degree of compaction, which can be assessed by measuring biophysical properties like mass density, and the morphology of MSph, differs from that of spheroids based on tumor cell models (sometimes called tumoroids), because tumoroids generally increase

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in diameter over time, whereas MSph do not. Moreover, the inner core in MSph may become senescent, but not necessarily necrotic, as is often the case in tumoroids.

By measuring the mass density of a viable spheroid, researchers can obtain quantitative information about the quality of their MSph culture and improve reproducibility in experiments. Indeed, it is important to generate MSph that display a specific range of mass density within a defined temporal window (days) in order to compare their quality, in terms of donor diversity among the same tissue sources, viability normalized by the seeded cell number at the initial formation of the spheroid, and the presence of functionally diverse clones within the typically heterogeneous MSC pool, as well as the level of senescence in the aggregate. Similar consideration is important in case of treatment of the MSph with non-toxic agents or checking the morphological and functional changes in MSph formation following the supplementation with a priming compound or drug.

Similarly to several 3D culture models, generated by using different modified culture conditions, we used ultra-low attachment, cell-repellent culture plates to obtain non-adherent homogeneous aggregates of MSCs. In this study, we formed spheroids originating from dispersed single MSC populations from different sources and measured the mass density using the W8 instrument, while also collecting cell metabolism data throughout the analysis.

2. Material and methods

2.1. Primary cell isolation

Isolation of Wharton's Jelly Mesenchymal Stromal Cells (WJ-MSCs, WJ) has been performed as previosly reported by Paris et al. (2023). Dental Pulp MSCs (DP) were isolated as previously published by Pierdomenico et al. (2005). Chorion MSCs (CH) were isolated as previously published by Pasquinelli et al. (2007). Adipose MSCs (ASC) were iolated as previoulsy published by Rossi et al. (2020). Amniotic membrane MSCs (AM), Bone marrow MSCs (BM) and fetal membrane- MSCs FM were isolated as previously published by Alviano et al. (2007). All tissues were originally collected after informed consent, according to the policy approved by the local Ethical Committee. For the experiments cryopreserved cells were thawed. Molecular characterization of the cells is described in Table 1.

2.2. Cell culture

After the isolation, all MSC types were cultured and expanded in growth medium that is DMEM low glucose, supplemented with 10% FBS, 2 mM glutamine and penicillin and streptomycin antibiotic solution, 5% CO_2, 37 °C. The cells were passaged before reaching 90% confluence. Trypsin or Accutase was used to detach the cells and passage them till the passage 10.

2.3. MSph formation

To generate non-adherent cell aggregates, we seeded 5000 cells in 200 μL of growth medium in a single well of a cell-repellent 96well-plate (Greiner Bio-One). The plate was centrifuged 50*g*, 3 min, to allow the seeded cells to settle and quickly make contact each other. 5% CO_2, 37 °C. Every 2 days, 150 μL of exhausted medium was removed and same amount of fresh medium was added to the well including the MSph.

2.4. W8 analysis

MSph were collected and fixed in 10% formalin after a single wash in DPBS. Before the analysis they were washed twice in DPBS and resuspended in 7 mL of W8 analysis solution. Each spheroid ranging in 50–500 μm diameter is analyzed for mass density, diameter and weight, setting robustness = 3 as a parameter of instrument accuracy.

2.5. CellTiterGlo assay

CellTiterGlo was purchased from Promega Corporation to quantify intracellular ATP content. Each Msph was collected and transferred to a single.96well-plate, in 100 μL of medium. Then 100 μL of CellTiter working reagent was added to each well and samples were agitated 150 rpm for 1.5 h, to ensure complete lysis of the cell clusters and cellular ATP release in the sampling volume.

Table 1
Immunophenotype of MSCs included in this study. CD, cluster of differentiation; -, no expression; +, expression.

Marker (antibody)	Expression
CD14	–
CD34	–
CD73	+
CD90	+
CD105	+

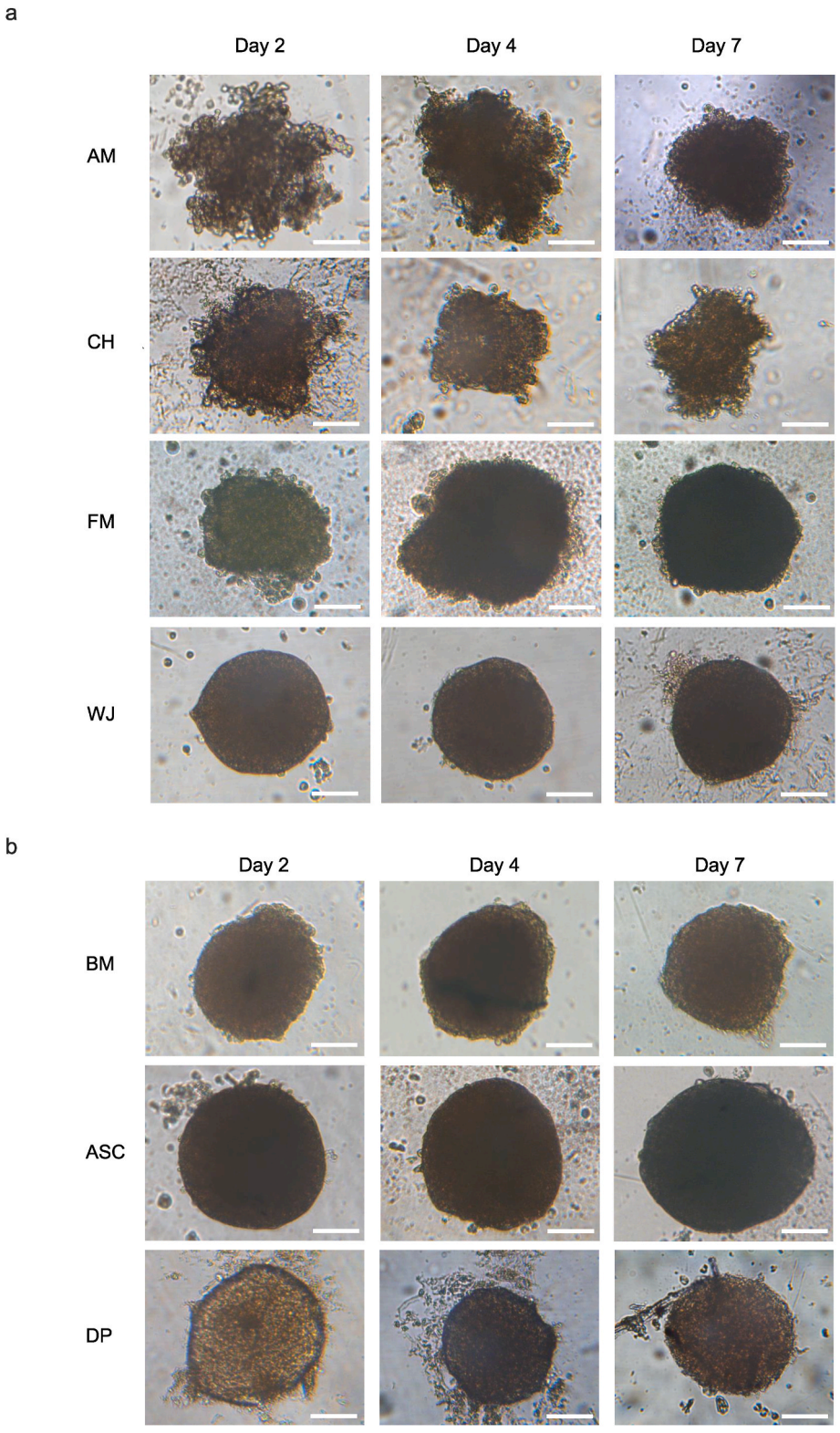

Fig. 1. Optical microscopy of the MSph.
Pictures of the perinatal MSph (a) and adult MSph (b) were taken after 2 days, 4 days and 7 days from cell seeding in non-adherent plate. Images for each MSph source were acquired during separate representative experiments. Each white scalebar is 120 μm.

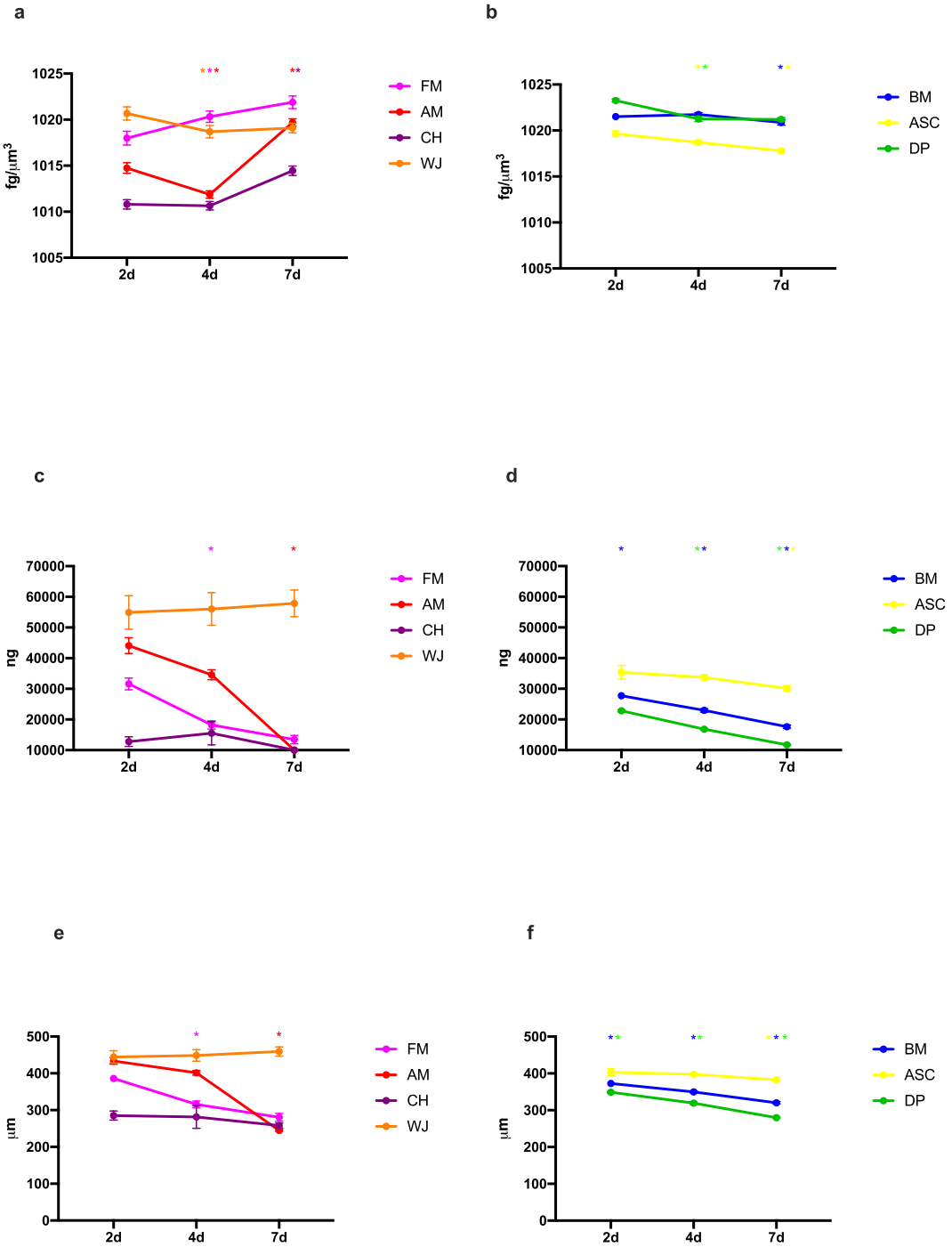

Fig. 2. W8 analysis of the biophysical properties of the MSph.
W8 analysis for measurement of mass density (a,b) weight (c,d) and diameter (e,f), respectively for perinatal MSph (a,c,e) and audlt MSph (b,d,f). Data are expressed as Mean ± SEM.Two-Way ANOVA test, $p < 0.05$ and LDS correction has been used to highlight significant differences among timepoints. The asterisk indicates a significant difference ($p < 0.05$) between the previous and the next time-point. For each time point at least 8 spheroids were analyzed. 2d, 4d and 7d stands for two days, four days and seven days from cell seeding in non-adherent plate.

Luminesce of at least 6 spheroids for condition was red at Victor 2 multiplate reader.

2.6. MSph treatment

For modeling natural compound supplementation, we selected Plumbagin (PB) molecule from *Plumbago indica*, which was purchased by Sigma Aldrich –Merck. Stock of 10 mM was prepared by diluting in DMSO, further dilutions were then prepared from the stock in DMEM. Cells or spheroids were exposed to 0.5 M PB for 24 h, 5% CO_2 and 37 °C. Both W8 analysis and CellTiterGlo assay were performed following the exposure to PB.

2.7. Statistics

For the experiments different donors were used for each MSC sources, at least two donors were used for experiment. Samples were analyzed and plotted using Prism GraphPad Software. T-test or One Way ANOVA methods have been used to define statistically significant differences. The P-values and minimal number of samples analyzed are indicated in the legends.

3. Results

3.1. Different sources of MSCs can form MSph

MSCs from both adult or perinatal source can form MSph within the first 48 h, despite differences in aggregate edges and shape (Fig. 1). Roundness is clearly higher for DP, ASC, BM and even perinatal WJ MSph, while placental membranes derived MSph, which are AM, CH and FM exhibit lower roundness.

3.2. Biophysical parameters of MSph are influenced by tissue source

The W8 instrument measured mass density as an indication of compaction ability of the MSCs in forming aggregates. Mass density changed significantly between the time points in MSph, however, this variation depended on the source and time points (Fig. 2). Perinatal MSph react different in the generation and compaction of MSph, while adult MSCs have mass density always higher than 1015 fg/μm^3 and with slighter variations among time-points. FM showed an increase after consecutive time-points days, in line with weaker ability of this source to quickly form MSph. WJ and DP display a similar trend, where the first days have the highest mass density, followed by a decrease on day 4 and remained the same at 7 days. For instance, WJ and DP presented values such as 1020 fg/μm^3 of a compact spheroid at 2d. The data presented showed a different panel for weight dimensions among all cell types, but at day 2, 5 cell types (all adult + 2 perinatal) out of 7 cell types weigh between an average of 20000-40000 ng. CH cells are lighter and do not show significant differences over time, while WJ cells are heavier than all other 6 analyzed cell types and do not change the weight also.

The perinatal WJ-MSph showed a particular trend, indeed they maintained stable parameters between day 4 and day 7 and a higher degree of compaction, comparable to the one of adult stem cells. WJ-MSph showed highest average weight among the MSC sources.

3.3. Viability of MSph decreased during 7-days in static suspension culture

CellTiterGlo signals showed that the quantity of ATP at the first timepoint (2 days from seeding) is higher for less compact spheroids, such as for the ones that are below 1020 fg/μm^3. However, MSph with mass density above 1015 fg/μm^3, such as AM/CH/DP-MSPh exhibited lower ATP signals in comparison to ASC-Msph or WJ-MSph (Fig. 3). Independently from the tissue source and MSC type, except BM-MSph, a strong decrease (≥50%) in ATP amount is detectable at the second time point, which is 4 days after the seeding for spheroid formation. An exception is FM-Sph, which showed no significant change in the ATP content between day 2 and

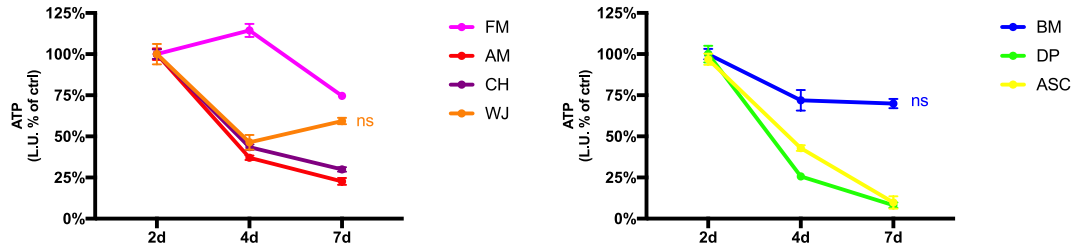

Fig. 3. CellTiterGlo analysis of the viability of the MSph.
Data are expressed as relative viability in comparison to the first timepoints. Dots indicated Mean ± SEM, Data express significant values between the previous and following timepoints, by One Way ANOVA test, $p < 0.05$, with the exception "ns" (not significant) indicating a not significant difference with the font colour indicating the relative source. For each time point at least 6 spheroids for donors were analyzed. 2d, 4d and 7d stands for two days, four days and seven days from cell seeding in non-adherent plate.

day 4. Again, similar to the trend recorded during W8 analysis of physical parameters, WJ-MSph have an increase at the day 7th for the ATP quantity.

3.4. The addition of a natural compound affects MSph biophysical parameters

FM is probably the MSC source that showed the best sensibility and greater variation of the biophysical parameters over several days, for this reason FM was chosen for testing how the supplementation with a compound can affect MSph properties. After detected a maximum dose of PB for which cell viability was not impaired after 24 h of treatment in FM monolayers (suppl. info), we performed a crystal violet assay to quantify cells based on DNA content. The treatment of FM-MSph with PB 0.5 μM did not increase DNA content

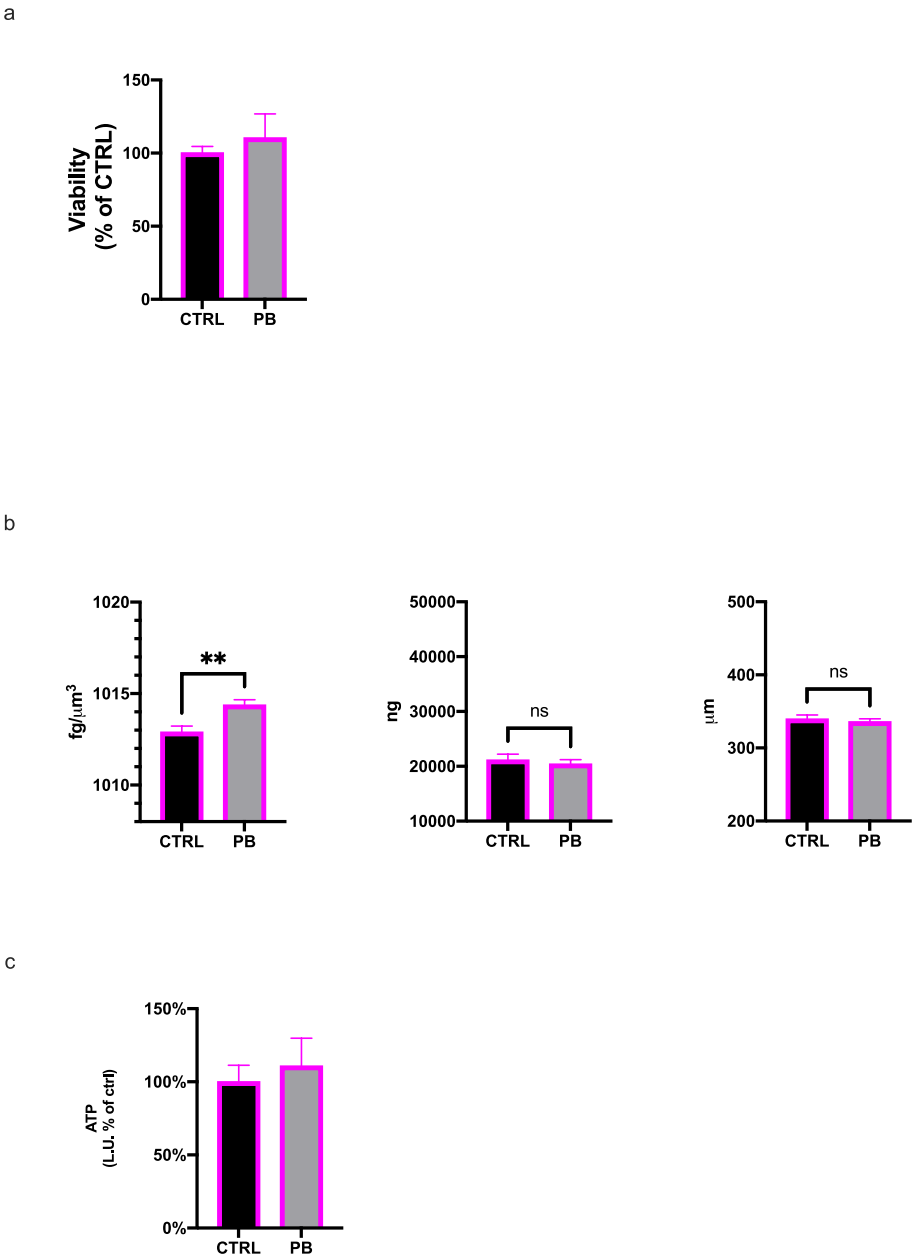

Fig. 4. Analysis of the viability and of the biophysical properties of the MSph exposed to PB.
a) Prestoblue assay for spheroid of FM-MSCs treated with 0.5 μM PB. The bars indicate Mean ± SEM. b) W8 analysis of PB-supplemented FM-MSph 3 days after seeding. The bars indicate Mean ± SEM. From left to right, graphs shows mass density, weight and diameter. c) CellTiterGlo assay for PB-supplemented FM-MSph for 24h. The bars indicate Mean ± SEM. CTRL, unsupplemented cells; PB, plumbagin supplemented cells. The asterisk denotes a statistically significant difference ($p<0.05$), as derived from T-test method.

after 4 days in culture, while PB 1 μM decreased it, thus hypothesizing a long-term cytotoxicity at higher concentrations (suppl. info). Based on these preliminary experiments, PB 0.5 μM has been selected as the treatment/priming dose for FM-MSph. To assess that 0.5 μm PB was not toxic in both 2D and spheroid cultures, FM-MSph were evaluated for cell viability by double strategy: first, the attachment to the plastic and second the conversion of resazurin into fluorescent resarufin, by incubating the metabolic-reactive reagent onto reattached spheroids. There was no cytotoxicity, as seen by similar reattachment to tissue-culture plastic surface in both control and PB-treated samples (data not shown). In addition, resarufin assay did not show decrease signals for PB-treated samples in comparison to controls (Fig. 4 a). The MSCs were not impaired in forming the standard intercellular connections that are essential for spheroid assembling even in presence of PB. FM-MSph formed by supplementation of PB were precisely detected by W8 (Fig. 4 b), highlighting any significant decrease in both weight and diameter of the spheroid. Similar results were obtained also using ASC-MSph (suppl.info). On the other side, PB changed mass density. The absence of a significative weight change should lead to the fact that treated MSphs have similar live cell number, but the intracellular space was reduced and spheroids result as more compact structures. For this reason, we checked the viability of FM-MSph forming in presence of PB, by sacrificing the MSph for CellTiterGlo assay but evaluating such MSph under the same conditions of W8 data acquisition. PB supplementation in MF MSph cultures did not alter viability (Fig. 4 c)

4. Discussion

The avenue of 3D cell culture for MSCs is critically linked to the goal of obtaining *ex-vivo* expansion as well as a greater number of more functional cells for clinical applications in regenerative medicine. We aimed to analyze the behavior of the simplest buildable 3D model, which can be cultured by aggregating MSCs spontaneously and without the support of a scaffold *in vitro,* a non-adherent, self-assembled spherical-shaped culture of floating multipotent cells called MSC-Spheroid (MSph). These models may overcome limitations associated with traditional MSC-2D cultures (Tietze et al., 2019). First called by (Méndez-Ferrer et al., 2010) as cells able to support the bone marrow hemopoietic niche, these mesenpheres were discovered as Nestin^{+} MSCs that can be propagated in cultures as non-adherent spheroids. Murine nestin^{+} BMSCs can form clonal mesenchymal spheres also called mesenspheres which can self-renew and differentiate into mesenchymal lineages (Isern et al., 2013)Human mesenspheres were described by the same group as aggregates derived from cells co-expressing nestin, CD146 and CD105 (and lacking CD45 expression). Other group indicates mesensphere arised from CD146^{+} Cd271^{+} sorted cells from bone marrow. Then, clonal spheres in nonadherent culture conditions were also designated as mesenspheres. More recently, in different reports, spheroid generally assembled by MSCs (Abbasi et al., 2018) and both murine and human MSCs (Bonilla et al., 2019) that can form spheroid with maintenance of MSC stemness were indicated by the same name. Since the nomenclature is not precise so far, we decided to call our spheroid formed with the same method from different sources as MSC-Spheroid or MSph. Our MSph are very similar to the murine MSC-spheroids, also called mesenspheres by Baraniak et al., in 2012 (Baraniak and McDevitt, 2012), since we wanted to start from MSCs and have a homogeneous population of MSC-spheroids of controlled size while maintaining multipotency for extended periods in suspension culture. They fulfilled immunophenotype MSC criteria and their biological properties, like mesodermal lineage differentiation and immunomodulation, were maintained (data not shown).

When compared to monolayer culture, the MSph exhibited superior biological functions such as paracrine activity, extracellular matrix (ECM) 3D organization, and greater differentiation potential, which are likely linked to enhanced cell–matrix interactions and intercellular connections. The intercellular adhesion and signaling pathways belonging to adhesion molecules signals in the ECM may have an expected important role in influencing stem cell fate.

The possible efficient retention, within the injection site, of the MSph would transplant endogenous ECM that could work as a native scaffold to increase engraftment and survival.

In our protocols, we tried to push the MSCs to mimic *in vivo* conditions like cells depositing ECM, in a 3D architecture established in a well static culture with a stable microenvironment; however, the natural dynamic spread of nutrients and gases, as *in vivo,* is almost missing except for the artificial manually media changes (as in 2D standard routine culture conditions).

After the seeding of MSCs in non-adherent condition, there is an establishment of complex cell-cell and cell-ECM dynamic interactions during the various stages of the time of culture. Although cell adhesion molecules such as integrins and cadherins have been implicated in the formation of cell spheroids (Lin et al., 2006) adhesion junctions and in particular cadherins play a central role in regulating the biophysical properties of MSph (Griffin et al., 2017; Shen et al., 2021; Zingales et al., 2023). Cadherins are fundamental players in spheroid formation because the homophilic cadherin–cadherin interaction contributes to switch from loose aggregates to more compact ones (Cui et al., 2017; Kang et al., 2024; Paris et al., 2023)

Considering the compact nature of spheroids and the role of junctional proteins, we opted to measure the biophysical properties of our cell aggregates using a specialized device called the W8.

W8 label-free microfluidic analysis of biophysical parameters is an advantageous technique because it is not destructive, thus this instrument can also work as quality control equipment for evaluating and sorting spheroids with different mass density or size made from MSCs (Marrazzo et al., 2021)

Our experiments indicate that the mass density of the MSph reanges between 1012 and 1024 fg/μm^3, depending onMSC source and their capacity to create stable aggregates. More compact MSph showed mass density similar to tumoroids, displaying values around 1020 fg/μm^3 (Sargenti et al., 2020). The W8 data were consistent. For example, what it has been obtained from adipose MSCs was in line with a previously reported mass density mean of about 1018 fg/μm^3(Sargenti et al., 2023).

At the first time point, there were substantial differences between cells derived from FM, AM, CH, which showed a lower mass density compared to other MSC sources. The weight differences shown in Fig. 2 likely reflect the differences in single cell diameter and

the inner content of molecules that each cell type can accumulate into the cytoplasm and make the cell heavy. WJ-MSph showed stable diameter over days and the highest average weight among the MSC sources, thus we hypothesize that ECM contribution to a larger structure and weight is more important for this cell type than for the others. This hypothesis is supported by the fact that WJ MSph present good viability maintenance, similar to BM-MSph which instead have some small decrease in weight and diameter. On the contrary, the ASC-MSph while did not change weight and diameter after 2 days, showed a progressive decrease, with very low signals at 7 days, in terms of viability over time. The DP-MSph decreased in all properties we followed, such as mass density, weight, diameter and also viability.

To summarize, there are sources of MSph that may be less feasible to produce viable, compact spheroids beyond 4 days of static culture, for example DP, ASC and AM. CellTiterGLO assay has been selected as a metabolic cell viability indicator instead of the non-lytic resazurin-bassed assay (e.g. AlamarBlue or Prestoblue) to overcome the limitation of the latter. In fact, resazurin viability dye should be pass through the very compact or less compact MSph and into their forming cells, before generating a detectable signal by fluorescence of the converted resarufin dye, but the compaction strongly influences the kinetics of this conversion, which happens from the spheroid structure to cell medium in which resarufin is reversed. In presence of MSph compaction diversity, resazurin-bassed assay is not suitable for comparing MSph, rather than a lytic assay in which mass density did not impact on the signal detected, because metabolic reaction is measured in MSph cells' lysate and not relying on the cell-trans-cell permeability of a dye. Measuring ATP in MSph cell lysate by CellTiterGLO assay we successfully and without limitations compared MSph regardless of variable mass density, thus we assessed viability of the cells during days for all cell sources regardless of different permeability of cell-cell interaction. Even though cell culture medium has been replaced with fresh medium every 2 days (at each time points for remaining not lysed samples), the metabolic rate of the MSph, as measure of ATP content, decreases overtime. This can reflect the limitation in removal of the cellular waste molecules before a static culture has been set up in our study; alternatively, the ATP loss in MSph after 2 days can sound as not technical issue, but as normal retention within the ECM of the MSph of the cellular waste that is not promoting the healthy cell state. The suggestion is to use for further application of the MSph samples cultured in static conditions for no longer than 3 days. Despite this general trend we showed that MSph made from FM MSCs had no significant change in ATP content between day 2 and day 4; this may be due to the slower aptitude of these cells type to form a compact spheroid, perhaps as a natural combination of MSCs derived from entire amnio-chorionic membrane. Interestingly, the WJ MSph may be able to have a rescue phase in which the cells try to survive not optimal culture in static conditions, indeed they increased at day 7 both diameter size and relative ATP-indicated viability.

Briefly, the compaction and the viability do not exhibit a unique correlation among the different sources; thus, the loss of viability may derive from specific donor properties or tissue origin ability to produce different content and quality of ECM, the one mostly influencing the weight of MSph. Also, all MSCs have a different metabolic activity, depending on the passage *in vitro* but mostly due to the tissue source original energetic rate (e.g. ATP production and use). It also be considered that MSph core can undergo to senescent state in different way according to donor and sources.

The priming of MSCs (Marrazzo et al., 2021) seems to be a very promising method to obtain *in vitro* expanded stem cells with enhanced immunomodulatory properties, as biological material suitable for cell therapy of inflammatory diseases and regenerative medicine applications. Usually, spheroids are tested with chemotherapic drugs as a model of chemoresistance, but this will not be a proper test for MSph. On the other hand, many natural bioactive compounds, as alternative to human chemokines and microbial components, can influence the biological properties of stem cells, based on various antioxidant or immunoregulatory mechanism. Each of these compounds should work in a non-toxic range of concentrations but may be used to prime or treat the cells of MSph. By reaching the cells forming the MSph, a natural compound may change morphology or phenotype of the MSCs then changing expected reference mass density record. For this reason, plumbagin (PB), a natural compound with several anti-inflammation potential (Petrocelli et al., 2023) has been tested as treatment of MSph. It is important that a non-toxic compound will not decrease mass density, as it has been demonstrated that the loose of mass density measured by W8 in tumor cell line derived spheroids is mostly an indicator of non-viable cells (Sargenti et al., 2020).

The treatment with PB showed no cellular toxicity in FM-Sph according to cell metabolism data and the compound was supplemented in culture and used to prime cells during MSph formation, as a model to monitor biophysical changes. Since an increase in cell number can result in a corresponding increase in mass density, a non-mitogenic dose of the selected compound was incubated. Interestingly, by supplementing with of PB, as a potential modulator of MSCs and priming strategy model, the mass density of FM-Sph changed in comparison to non-supplemented MSph formed controls, after 3 days following the seeding. These results are consistent with the modification of MSph aggregation profile by some biological activity of PB, which are still under investigation. On the other hand, the weight and diameter of PB-supplemented FM-Sph did not change, due to successful compound permeability into MSph and possible temporary bioactive effect.

5. Conclusions

Tracking the progression of 3D cultures can evolve into a primary approach for developing optimal MSph for future applications. In this context, the possibility to assess the quality of 3D stem cell-derived culture by biophysical parameters that determine their growth and uniformity can be a key approach to increase the reproducibility of MSph generation for future demanded clinical applications. The suspension culture in which the MSph are generated is suitable for bioreactors in cell-culture factories, for allowing future production of cellular products. In such comparative study, we highlighted some raw properties of MSph while demonstrating the limitations of their static condition culture that should be confined to a short-term culture, to preserve the suitability of MSCs for differentiation protocols or further protocols before delivering them. Since MSC spheroids constitute a relatively unexplored research field, more research is encouraged to understand additional properties of MSph and similar culture models, to reveal their full

suitability for transplantation and regenerative medicine strategies.

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CRediT authorship contribution statement

P. Marrazzo: Writing – original draft, Methodology, Investigation. **A. Sargenti:** Writing – review & editing, Investigation, Data curation. **R. Costa:** Writing – review & editing, Data curation. **F. Paris:** Formal analysis. **J. Peca:** Investigation. **D. Piras:** Validation. **V. Pizzuti:** Formal analysis. **S. Pasqua:** Project administration, Conceptualization. **F. Alviano:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

Azzurra Sargenti reports a relationship with CellDynamics iSRL that includes: employment.

Davide Piras reports a relationship with CellDynamics iSRL that includes: employment. D. Piras has been PhD student at University of Sassari.

Simone Pasqua reports a relationship with CellDynamics iSRL that includes: employment.

Francesco Alviano reports financial support was provided by CellDynamics isrl. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbior.2024.101052.

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