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Comparative analysis of cryopreserved adipose stem cells expanded in hollow fiber bioreactor versus conventional tissue culture flasks

Guoqiang Ren[1], Morten Brøndum Sørensen[2], Simone Riis Porsborg[2], Trine Fink[2], Vladimir Zachar[2] & Qiuyue Peng[2]✉

Cryopreservation enhances the availability of "off-the-shelf" cell therapies. However, the choice between tissue culture polystyrene (TCP) and hollow fiber system (HFB) system for adipose-derived stem cell (ASC) production remains a critical decision, with implications for scalability, reproducibility, and the clinical efficacy. Therefore, the characteristics of ASCs expanded in TCP and HFB and cryopreserved were compared. TCP and HFB cultures were established, and cells were cryopreserved. Surface markers were analyzed to identify immunophenotypic changes and subpopulations. Clonogenicity, differentiation capability, and proliferation potentials were determined along with surrogate tests on wound healing. The expressions of the most markers were consistent before and after thawing for both systems. However, CD105 expression of TCP cells was significantly decreased by the freeze-thawing procedure. Also, CD274 was significantly less expressed on HFB-expanded cells before freezing, however, post-thawing, the proportion of CD274 positive cells was comparable to TCP cells. Besides, two expansions supported different subpopulations, influencing the heterogeneity within ASC cultures. Despite this heterogeneity, no statistical differences were observed as for ASC functional characteristics and the effects on fibroblasts. This study highlighted freeze-thaw does not interfere with the production of fully functional ASCs in either system, although it drives some differential changes in the subpopulations between systems.

Keywords Cryopreservation, Adipose-derived stem cells, Hollow fiber bioreactors, Tissue culture polystyrene, Flow cytometry, Subpopulations

Adipose-derived stem cells (ASCs) have emerged as promising candidates for diverse applications in cell-based therapies, ranging from dermatology and aesthetic rejuvenation, COVID-19 pneumonia, wound healing or ischemic heart disease, among others[1]. Compared to other types of stem cells, the benefits of ASCs include readily accessibility, higher abundance, lower immunoreactivity, and the absence of ethical concerns[2]. To ensure the effectiveness of ASC therapies, multiple infusions are often required, necessitating a substantial quantity of cell samples[3]. Currently, both tissue culture polystyrene (TCP) flasks, and hollow fiber bioreactor (HFB) systems are used to expand necessary cell numbers and cryopreservation of cells allows for off-the-shelf products[4]. During both expansion and cryopreservation, maintenance of cell characteristics is necessary for optimal bench-to-bed side translation; and monitoring of cell characteristics is mandatory for clinical products.

It is well-known that flask and bioreactor cultures have their advantages and limitations. TCP-based cultures are favored for their cost-effectiveness and versatility, making them accessible for smaller-scale research endeavors. However, they are labor-intensive with manual handling requirements that can lead to variability and hinder scalability[5]. On the other hand, automated HFB excel in scalability and consistency, making them ideal for large-scale cell manufacturing and applications demanding reproducibility. Nevertheless, their high initial costs, technical complexity, and the need for specialized expertise can be limiting factors, particularly for smaller research facilities[6,7]. The selection between these two approaches depends on a careful balance between resource

[1]The Affiliated LiHuiLi Hospital of Ningbo University, Ningbo University, Ningbo 315040, China. [2]Department of Health Science and Technology, Aalborg University, Gistrup 9260, Denmark. ✉email: qp@hst.aau.dk

availability, scalability requirements, and the desired level of experimental control, highlighting the need for a dedicated decision-making process in designing cell-based studies or therapeutic production processes. While the choice of expansion methods is often determined by these practical considerations, care must be taken to clarify the influence of expansion systems on ASC characteristics. In addition, as most current clinical protocols call for the use of cryopreserved cells, the extent to which the freeze-thaw cycle alters the characteristics of ASCs obtained from these two production methods should be explored.

In this project, we aim to undertake a thorough investigation of the impact of the freezing-thawing process on ASCs produced by two different systems, specifically assessing changes in immunophenotypes, stemness, viability, proliferation, and effect on fibroblasts' migration. Importantly, as the phenotypic profiles of the ASCs change along with cell proliferation[8,9], we have ensured that the experimental setup of this study allows for comparison of TCP- and HFB-expanded ASCs that have undergone essentially similar population doublings, even though each expansion system necessitate quite different passaging schedules. This study contributes valuable insights into the selection of clinical-grade manufacturing approaches.

Results

Surface epitope changes

To ensure comparable population doublings between the HFB and TCP systems, one-fifth of ASCs were seeded in a HFB (1.7 m^2) system for a single passage, while fourth-fifth ASCs (equivalent to a quarter of HFB-cells) were seeded into a single T175 TCP flask (0.175 m^2) and expanded 1:3 until P4, in theory yielding 27 T175 flasks (totaling 0.47 m^2, a quarter of HFB surface area), as depicted in Fig. 1. However, for practical consideration, only one-third of TCP cells were continued in each subsequent passage. HFB cells at P1 and TCP cells at P4 were cryopreserved. Phenotypical analysis was performed before and after cryopreservation. Furthermore, for the cryopreserved cells, which are clinically relevant as an off-the-shelf product, functional tests were conducted to confirm the stem cell characteristics.

When analyzing the expression of single surface markers on ASCs, the expression of CD73 and CD90 was found to be highly expressed (> 95%) in both systems before and after freezing (Fig. 2A). Before cryopreservation, CD105 expression was over 95% for both systems, however, after freeze-thawing procedure, only 75% of TCP cells remained positive, resulting a significant difference between systems. For the markers analyzed in Panel B, CD29, CD201, CD36, CD31, and Stro-1, no significant differences were found, neither when looking into the effect of freezing nor the effect of the expansion system (Fig. 2B). However, it was observed that CD29 and CD201 were expressed on nearly 100% of the ASCs, CD36 and CD31 on < 9% of the cells, and that the expression of Stro-1, expressed in ~ 10% of ASCs (Fig. 2B). For Panel C, the expression of CD166 and CD200 was consistent, at 99% and around 3%, respectively. The expression of CD248, CD271, and CD146 displayed a concurrent, however insignificant trend, with the proportional expression of CD248 and CD271 increasing and the proportional expression of CD146 decreasing for the TCP system (Fig. 2C). Both CD34 and CD274 showed a differed pattern with freezing increasing the difference between systems for CD34 and decreasing the difference between systems for CD274. Actually, the expression of CD274 increased significantly by nearly 48%, balancing out the significant difference observed between the systems before freezing (Fig. 2C).

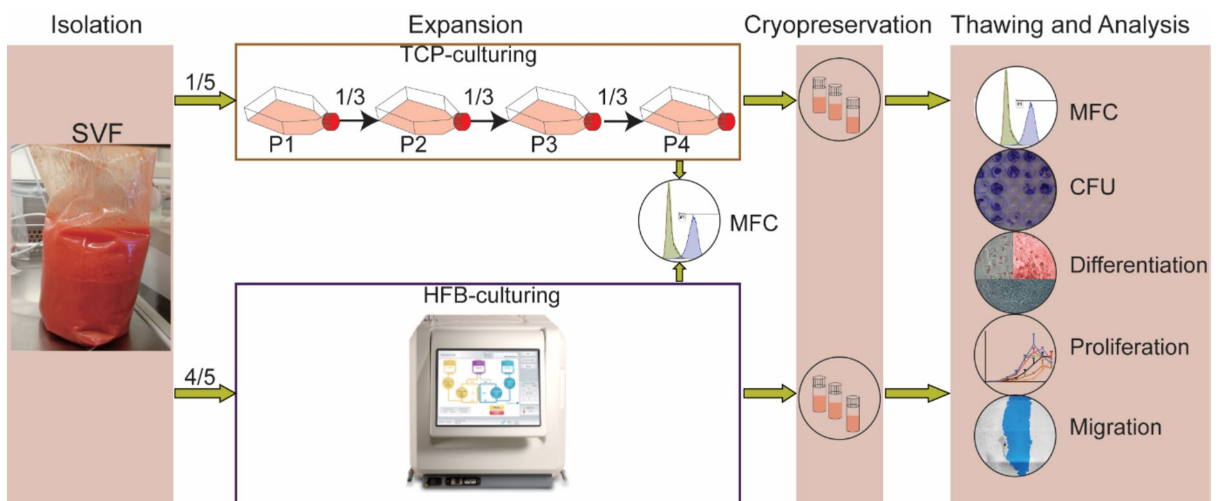

Fig. 1. Experimental scheme. One fifth of SVF was seeded into TCP flasks and passaged 1:3 when confluent until passage 4, while the rest of cells were cultured in the HFB system and harvested after one passage. Before cryopreservation, cells were analyzed by MFC, while post-thawed cells underwent both phenotypical and functional analysis. The whole procedure was performed independently for three3. *TCP* tissue culture polystyrene, *HFB* hollow fiber bioreactor, *SVF* stromal vascular fraction, *P* passage, *MFC* multi-color flow cytometry, *CFU* colony-forming unit.

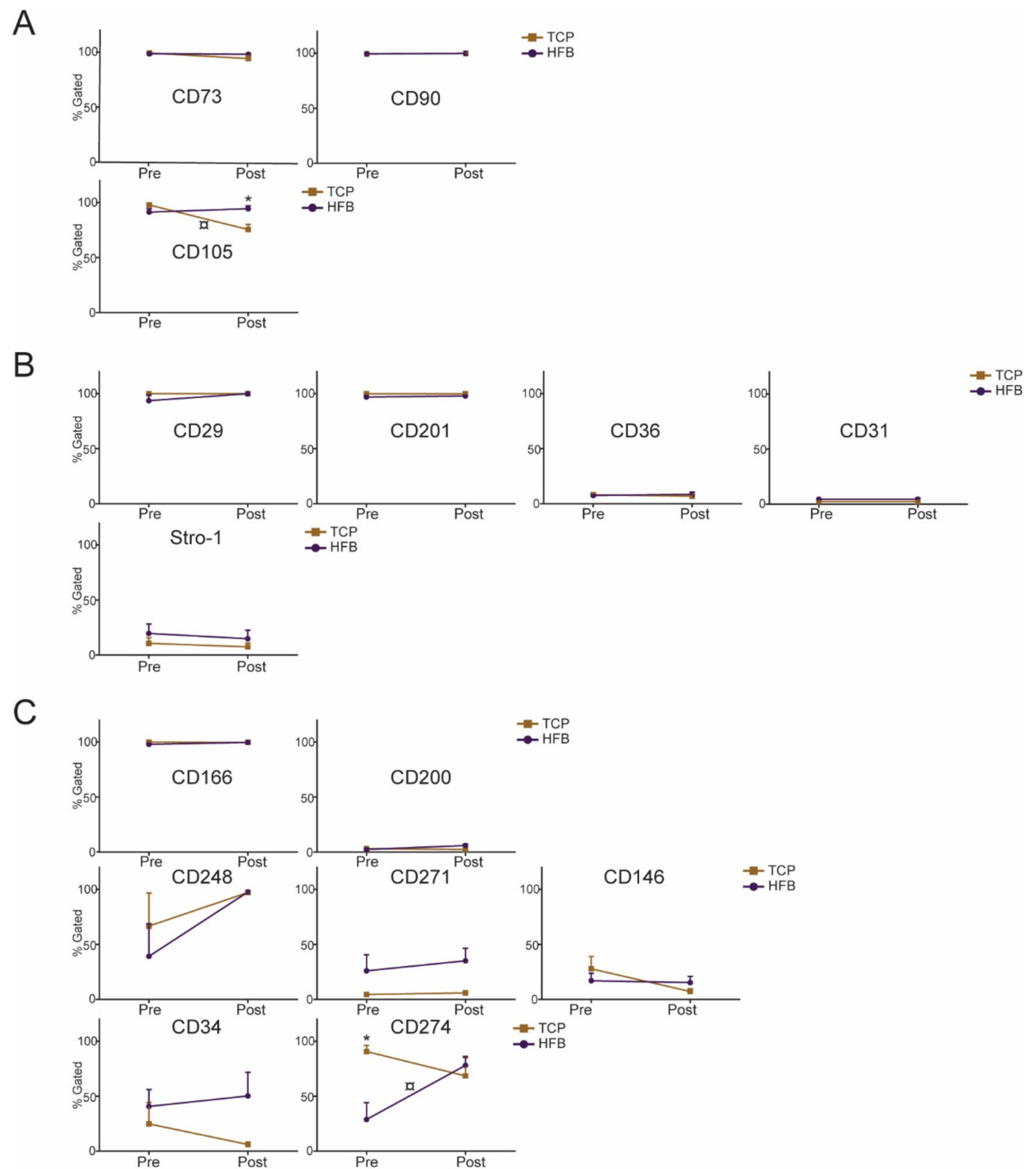

Fig. 2. The effects of freezing-thawing process on individual surface markers for both TCP and HFB system. (**A**) Panel was composed of CD73, CD90, and CD105. (**B**) Panel was composed of CD29, CD201, CD36, CD31 and Stro-1. (**C**) Panel was composed of CD166, CD200, CD248, CD271, CD146, CD34, CD274. Data are shown as mean + SEM from three donors. *Denotes significant difference between culture systems, $p < 0.05$. ¤Denotes significant difference post-thawing compared to pre-freeze, $p < 0.05$. *TCP* tissue culture polystyrene, *HFB* hollow fiber bioreactor, *Pre* pre-freeze, *Post* post-thawing.

Immunophenotypical subpopulation changes

When analyzing the co-expression of surface markers on ASCs, only meaningfully expressed subpopulations (> 5%) were explored. For Panel A, this was two subpopulations (SP), SPA1 and SPA2, with the expressional profiles being CD73+, CD90+, CD105− and CD73+, CD90+, CD105+, respectively. For SPA1, no significant difference was found between the two expansion systems before freezing; however, after thawing, a significant increase for the TCP system resulted in a significant difference between the two systems. For SPA2, a significant difference was observed between the two expansion systems before freezing, and as SPA2 significantly decreased in TCP cells after thawing, this difference between systems became even larger (Fig. 3A). When comparing the development of SPA1 and SPA2 between systems, the complementary change could be attributed to the apparent loss of CD105 in the TCP system (Fig. 3A). Regardless of the expansion methods or detection points, the triple positive population was by far the most abundant.

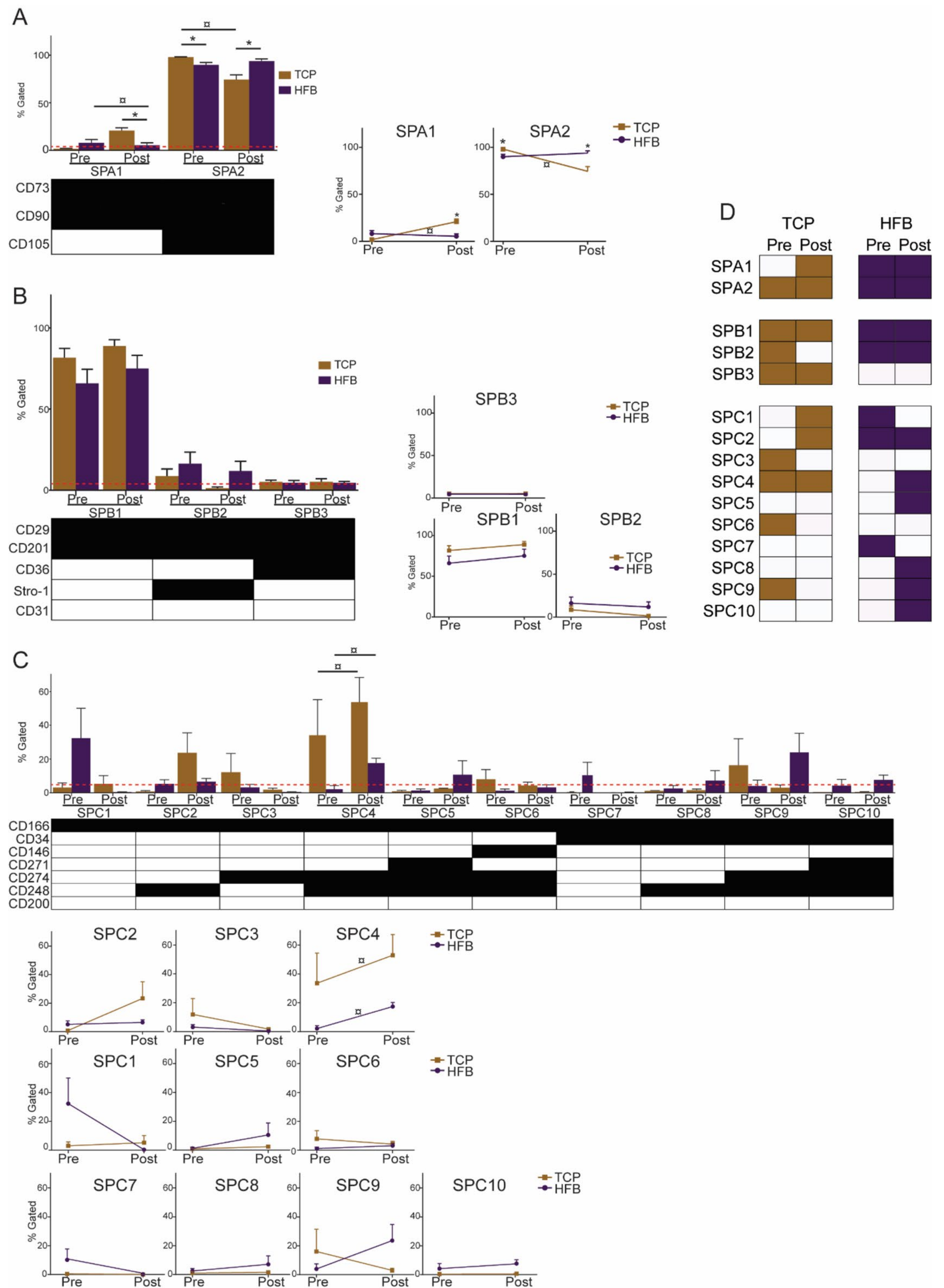

Fig. 3. The effects of freezing-thawing process on subpopulation profiles for both TCP and HFB system. Only subpopulations with occurrence higher than 5% on average were selected. (**A**) Subpopulation profiles were defined by CD73, CD90, and CD105. (**B**) Subpopulation profiles were defined by CD29, CD201, CD36, CD31 and Stro-1. (**C**) Subpopulation profiles were defined by CD166, CD34, CD146, CD271, CD274, CD248, and CD200. (**D**) Heatmaps showing the presence of subpopulations. Data are shown as mean + SEM from three donors. *Denotes significant difference between culture systems, $p < 0.05$. ¤Denotes significant difference post-thawing compared to pre-freeze, $p < 0.05$. *TCP* tissue culture polystyrene, *HFB* hollow fiber bioreactor, *SPA* subpopulation defined by (**A**); *SPB* subpopulation defined by (**B**); *SPC* subpopulation defined by (**C**); *Pre* pre-freeze, *Post* post-thawing. Black box denotes the presence of a specific surface marker, and empty box indicates its absence.

For Panel B, three subpopulations were found, SPB1, SPB2, and SPB3 with the expressional profiles being CD29+, CD201+, CD36−, Stro-1−, CD31−; CD29+, CD201+, CD36−, Stro-1+, CD31−; and CD29+, CD201+, CD36−, Stro-1−, CD31 + respectively (Fig. 3B). No statistical significance effect of either expansion system or cryopreservation was observed.

For Panel C, 10 meaningful subpopulations were identified, all positive for CD166 and negative for CD200, and further defined by the varying presence of CD34, CD146, CD271, CD274, and CD248 (Fig. 3C). Both SPC2, 3, and 4 changed their proportion in the same pattern, with SPC2 and SPC4 increasing after thawing and SPC3 decreasing (Fig. 3C). However, only the development of SPC4 was significant and applicable to both systems, with the highest proportion of this clone found among cells expanded in the TCP system. SPC4 is defined as CD166+, CD34−, CD146−, CD271−, CD274+, CD248+, CD200−. For SPC1, SPC6, and SPC7, even though insignificant, the effect of cryopreservation decreased the difference between systems. For SPC5, SPC8, and SPC10 the difference between systems increased without significance. Interestingly, for SPC9 TCP had the highest expression before freezing and HFB after thawing. An overview of the meaningfully expressed subpopulations is shown in the heatmap (Fig. 3D). It is evident that the different culture conditions favor distinct subpopulations and that TCP-expanded cells became less variable and HFB-expanded cells more variable during the freeze-thaw process.

Stemness and proliferation

The trilineage differentiation capacity, CFU, and proliferation were examined directly after thawing, while viability was assessed both pre-freeze and post-thaw (Fig. 4). After thawing, ASCs expanded in both systems demonstrated their capability to differentiate into adipocytes, osteoblasts, and chondrocytes, as evidenced by positive staining with Oil Red O, Alizarin Red S, and Alcian Blue, respectively (Fig. 4A). Control cells, maintained in the corresponding complete medium, did not exhibit such differentiation. Although HFB-cells appeared to have a higher colony-forming potential, we could not demonstrate a statistical significance (Fig. 4B). While both manufacturing methods yielded a cell survival rate exceeding 90% post-freeze-thaw, the TCP cells demonstrated a greater robustness than the HFB-cultured counterparts (Fig. 4C). As for the growth kinetics, no significant difference was found between the two types of cells (Fig. 4D). Of note, the DNA content decreased in the later stage of culturing (from 11 days of culturing). Upon reaching overconfluent. cells started to detach from the culture surface due to excessive crowding, waste accumulation or nutrient depletion, etc.

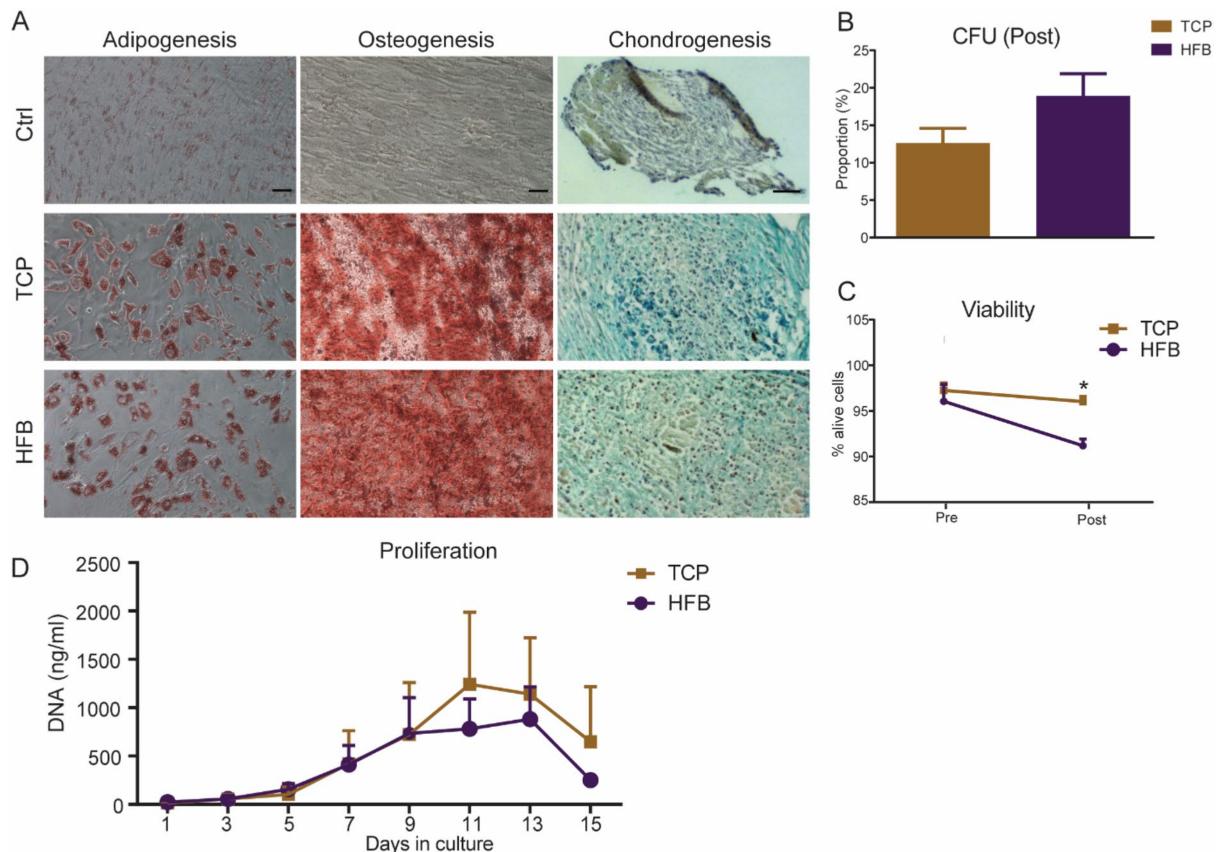

Fig. 4. ASC characteristics after expansion on TCP or HBF and cryopreservation. (**A**) Tri-lineage differentiation. Scale bar = 100 µm. (**B**) Clonogenicity. (**C**) Viability. (**D**) Proliferation. Data are shown as mean + SEM from three donors. *$P < 0.05$. *TCP* tissue culture polystyrene, *HFB* hollow fiber bioreactor, *Pre* pre-freeze, *Post* post-thawing, *CFU* colony-forming unit, *Ctrl* control.

Wound healing potential

In assessing biological assays pertinent to the granulation phase of wound healing, fibroblast migration and proliferation assays were employed. Specifically, a wound scratch assay was utilized to assess the influence of ASC paracrine effects on HDF migration, and a proliferation assay to examine the influence on HDF growth (Fig. 5). Representative images of the scratch closure (Fig. 5A) did not reveal any discernible differences, consistent with the results of quantitative evaluations (Fig. 5B). As for the HDF proliferation, no significant distinctions were observed between ASCs expanded in the two systems as well, both exhibiting a similar growth rate when cultured with ASC-CM, as illustrated by Fig. 5C.

Discussion

Numerous clinical trials are currently being conducted to ascertain the therapeutic effects of ASCs as a cellular therapy for various diseases. Standardization of the manufacture procedures and accurate characterization of the cellular product to ensure patient safety has become a paramount concern for regulatory agencies governing the approval of biological license applications[10]. Currently, the manufactures of ASCs involve flask-based or a closed bioreactor system, followed by cryopreservation to enable large-scale production for allogeneic off-the-shelf applications.

There has been considerable interest in using lower amounts of DMSO for cell therapy products, particularly in protocols where cells are not washed prior to injection. We chose to use DMSO at a low concentration (5%) as the cryoprotectant, which has been shown to preserve the viability and stem cell characteristics of mesenchymal stem cells[11–13]. Viability is a critical release criterion for clinical administration, with a minimum benchmark of 70% viable cells considered essential[14]. Here, both conditions demonstrated remarkable viability, maintaining over 90% of living cells following cryopreservation. However, the potential impacts of accumulated stress, which may continue to trigger cell death pathways in the hours to days after thawing and lead to delayed cell death[15], need to be considered. Given the time gap between thawing and eventual transplantation and taking effect in a patient's body, assessing this time window becomes particularly significant. To address this concern, we conducted a proliferation assay to investigate whether there was a delayed adverse effect on cell growth. The similar growth rates observed between the two manufacturing methods suggest that the detrimental effects of cryopreservation were not discernible in this context. Consistent with this observation, we did not observe any effects of expansion methods on either CFU or capacity for tri-lineage differentiation.

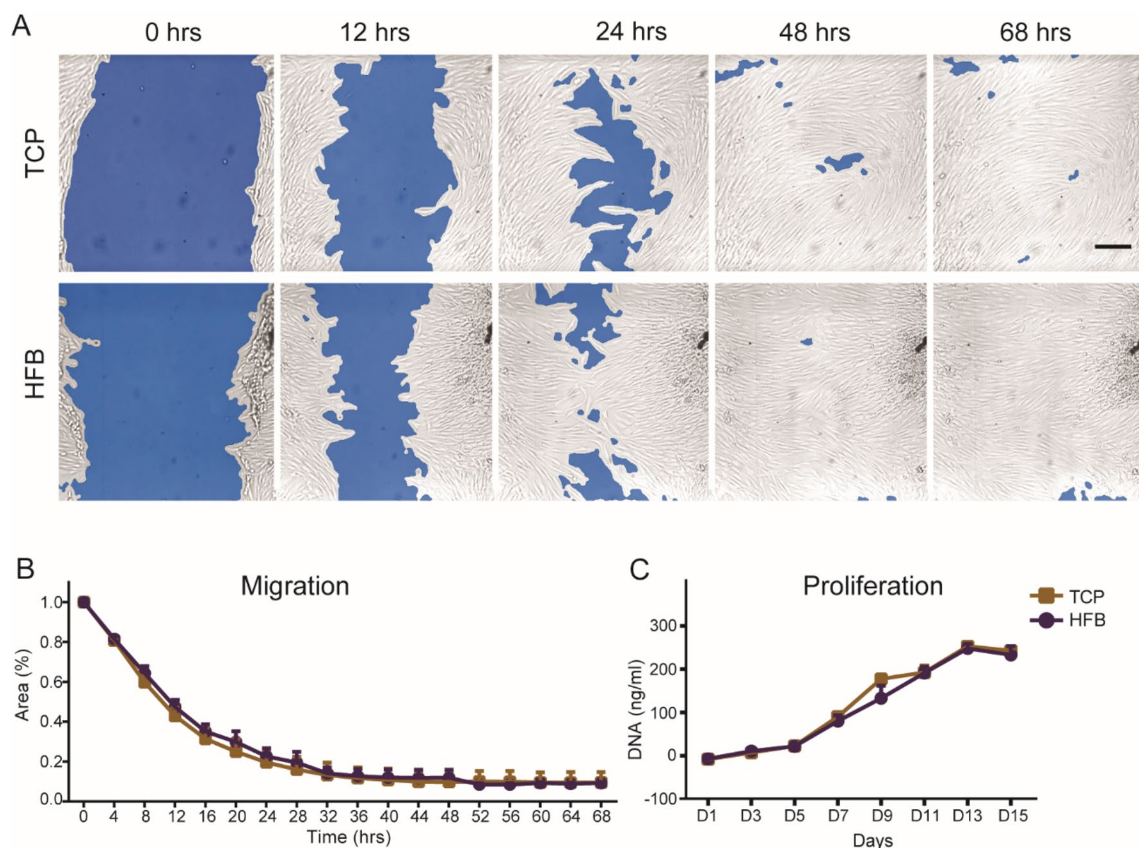

Fig. 5. Effects of ASC secretome on fibroblasts. (**A**) Fibroblast migration measured by scratch assay. Blue color represents cell-free area. Scale bar = 100 µm. (**B**) Quantitation of cell-free area over time. Data are shown as mean + SEM from three donors. (**C**) Fibroblast proliferation measure by DNA content from three donors. *TCP* tissue culture polystyrene, *HFB* hollow fiber bioreactor, *D* day, *Hrs* hours.

It has been hypothesized that freezing-thawing process effectively preserves the characteristics of ASCs, irrespective of the chosen expansion methods[16]. However, in this study, we found in fact that both culture methods and cryopreservation affect the ASC heterogeneity in terms of certain surface markers. The surface markers have previously selected based on their proposed association with mesenchymal stem cell properties (CD73, CD90, CD105), ASC identity (CD146, CD34, CD31), wound healing (CD166, CD248, CD271), immune regulation (CD29, CD200, CD274), and differentiation capacity (CD36, CD201, Stro-1)[8]. When looking at the multiparametric analysis, we observed a higher number of SPs in TCP-cells than HFB-cells before freezing and a lower number of SPs after thawing. This observation was mainly due to changes in the expression of only three markers, CD105, CD248, and CD274. The number of immunophenotypical subpopulations, determined by combinations of surface markers, reflects the heterogeneity of ASCs. A higher number of subpopulations indicates greater heterogeneity, which may complicate clinical outcomes. Including more markers allows for stronger and more definitive conclusions. In this study, post-thawing analysis revealed fewer HFB subpopulations compared to TCP, suggesting that HFB cells are more homogeneous. This finding supports the potential of HFB cells to align better with therapeutic efficacy expectations. However, this hypothesis requires further validation through comprehensive functional testing.

Undoubtedly, hallmarks confirming ASC identity, CD73, CD90, and CD31, were consistently positive or negative in both conditions throughout the entire process, consistent with previous studies[12,17]. Also, most of the other markers were not affected either. Unexpectedly, while the most of ASCs were positive for CD105, we found a significant decrease in CD105 expression on TCP-cells, in contrast to a report of robust CD105 expression post-cryopreservation[18]. This change of CD105 explains the transition of SPA2 into SPA1. Levi et al. reported that CD105 depletion could enhance ASC osteogenesis[19], prompting the inference that a loss of CD105 might be associated with enhanced osteogenic capacity for TCP cells. Notably, we opted to use HPL as the supplement, rather than the traditionally used fetal bovine serum/fetal calf serum (FBS/FCS), due to the concern related to potential zoonotic pathogens and immunogenic reactions[20], which could explain the divergence in relation to the previous study. In addition to reducing risk of pathogens transmission, HPL contains cytokines and growth factors which support ASC growth and genomic stability[21,22]. Consequently, more researchers have proposed HPL as a suitable serum substitute for cell therapy[23,24]. However, our study indicates the transition from FCS to HPL may introduce ASC phenotypical changes, which should be explored before in vitro findings can be translate into clinical effects. Although the expression of most of the markers were not affected by the expansion system, it appeared that culture in HFB was favorable to the expansion of CD274 negative cells. However, the presence of CD274 negative cells was reduced during the freezing-thawing process. Interestingly, we observed a higher cell death for the HFB-expanded cells, suggesting that these particular cells were more sensitive to this process. In addition, the CD274-negative cells may represent a subset with reduced immunoregulatory function. However, these functions need to be tested in future studies through additional sorting of the single populations and direct comparisons between the culture systems. Furthermore, the changes of CD248 were in line with the tendency observed from our previous studies on TCP expansion[25], but were obscured by large donor variations.

The triple positive (CD73+, CD90+, CD105+), can be considered primitive, as these cells can be found in the SVF of freshly isolated ASCs. In our previous study[8], we examined the dynamic changes of immunophenotypic subsets during in vitro culture on TCP through eight passages. Through such design, we were able to clearly identify the permissive (early-stage) and differentiated (late-stage) populations. However, in the current study, we only harvested cells after one passage for HFB culturing and four passages for TCP culturing, making it challenging to accurately identify permissive and differentiated populations. To address this question, an analysis of the subpopulation composition in both primary and dynamically cultured cells is necessary for a direct comparison of their presence.

This study provides an insight into the complex composition and shifting patterns of ASC subpopulations between the expansion systems throughout freeze-thawing cycle. The immunophenotypical subpopulations defined by variations in expression of the markers, could reflect distinct functional subsets of ASCs. For example, the presence or absence of the markers CD31, CD29, CD201, CD36, and Stro-1, may reflect changes in the differentiation, or immune regulation capacities of ASCs. Their dynamic expression may further indicate the acquisition or loss of specific functions of cells harvested from these two culture systems. However, this hypothesis needs to be validated through direct sorting of the populations and further comparative analysis.

While the identification of the major subpopulation determined by the cell surface markers is a valuable approach for charactering cell products in clinical applications; the immunophenotype alone cannot fully reveal the potency of cell products. Future work should focus on linking detailed surface marker profiles with the cells' biological potency to provide a more comprehensive comparison between the two expansion systems.

With the appearance of new therapeutic indications for stem cell therapy, it has become increasingly important to supplement classic characterization with functional assays that reflect the desired biological impacts. For instance, HDFs models can be relevant for wound healing. In this study, although differences in clonal distributions were found between expansion methods, these differences did not translate into biological effects on HDFs.

Conclusions

In conclusion, the expressions of most of surface markers, clonal capacity, and the tri-lineage differentiation, HDFs activation remain constant across culture conditions and during cryopreservation. While the expression of surface markers, CD105, CD248, and CD274, was influenced by both expansion methods and freezing-thaw cycle and thus altering the clonal distribution, the biological relevance of these changes is not yet clear.

Methods

Lipoaspirate collection and SVF isolation

A total volume of 100–150 ml of lipoaspirates were collected from three healthy donors who underwent cosmetic surgery (from Aleris-Hamlet Private Hospital and Aalborg University Hospital, Aalborg, Denmark). All donors signed an informed consent. Ethical approval for this study was granted by the regional committee on biomedical research ethics in Northern Jutland (Project no. N-20160025). Tissue collection complied with the principles defined by the Declaration of Helsinki and followed the rules defined by Danish legislation on anonymized tissue (Komitélov Sect. 14). From the lipoaspirates, the stromal vascular fraction (SVF) was isolated according to the previously established protocols[8,25,26].

TCP culturing

One-fifth of the SVF was allocated to TCP-T175 flask culturing and cultured in growth medium composed of alpha-Minimum Essential Medium with low glucose and GlutaMAX (Gibco) supplemented with 5% heparin-free PLTGold® human platelet lysate (HPL, Sigma-Aldrich, Søborg, Denmark) and 1% antibiotics (Gibco). Upon achieving a confluence level of approximately 80–90%, cell detachment was performed using TrypLE (Gibco, Taastrup, Denmark), and the subsequent cultures were initiated by using one-third of the cells released from the previous passage. The cells were passaged four times (P4).

HFB culturing

For preparation of the HFB, the procedures followed the manufacturer's instructions and previously published study[27]. In brief, a disposable cell expansion set (Terumo BCT, Lakewood, Colorado, USA) was loaded and primed with phosphate-buffered saline (PBS) to remove air from the fibers. The surface area of the fibers was coated overnight with cryoprecipitate (Blood Bank, Aalborg University Hospital) and subsequently washed with growth medium.

The remainder 4/5 of the SVF suspension was re-suspended with 100 ml growth medium, transferred to the Quantum cell inlet bag, and automatically loaded into the HFB under standard ASC culture conditions with an incubation temperature of 37 °C and a pre-mixed gas supply (20% O_2, 5% CO_2, balanced with N_2). Cells were cultured at a relatively static condition for 24 h without the inlet flow to ensure full attachment. Subsequently, the fresh medium was fed continuously at 0.1 ml/min. The non-adherent cells were washed away after three days of cultivation. Afterwards, the culture medium was continuously supplied at an increasing rate from 0.1 ml/min to 1.6 ml/min, maintaining the lactate level within the range of 6 to 10 mM. Lactate and glucose levels were measured on a daily basis (LactatePlus Meter, Nova Biomedical, Waltham, and ContourNext Meter, Ascensia Diabetes Care, Parsippany, both USA). When the flow rate reached 1.6 ml/min, and the lactate concentration stayed above 6 mM for 24 h, cells were released from the fibers by the addition of 180 ml TrypLE for 15–20 min and then collected inside a harvest bag (P1). The cell yield was determined using a Nucleocounter NC-200 (Chemometech, Allerod, Denmark).

Cell freezing and thawing

Cells harvested from TCP P4-culturing and HFB P1-culturing were resuspended in cryomedium composed of 5% dimethyl sulfoxide (Sigma-Aldrich) and 95% HPL. The cell concentration was adjusted to 2 or 5 million per cryovial (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) for TCP and HFB cells, respectively. Cells were cryopreserved in a − 80 ℃ freezer for 24 h in styrofoam box and then transferred to liquid nitrogen until use. After 2–3 months, cells were retrieved, thawed in running warm water, and analyzed.

Multi-color flow cytometry

The percentage of live cells and expression of surface markers, including CD29, CD31, CD34, CD36, CD73, CD90, CD105, CD146, CD166, CD200, CD201, CD248, CD271, CD274, and Stro-1, were analyzed on single cells using fixable viability dye (FVS570) and a batch of directly conjugated antibodies (BD Biosciences, Lyngby, Denmark). The panel design and related procedures, including compensation, staining, and data analysis, were conducted as previously described[28].

Clonogenicity

Four limiting dilutions were set at 1, 3, 10, and 30 cells per well, each in 12 replicates. After 14 days, cells were fixed with 4% formaldehyde (AppliChem, Esbjerg, Denmark), stained with 0.05% crystal violet (Sigma Aldrich), and scored by 2 investigators. Data complying with the Poisson distribution was included, and the clonogenicity analyzed by the L-Calc software (Limiting Dilution Analysis Software, Version 1.1, Stem Cell Technologies, Vancouver, Canada, https://www.stemcell.com/products/l-calc-software.html).

Differentiation assay

ASCs were analyzed for adipogenic, osteogenic, and chondrogenic differentiation capacity using StemPro differentiation kits (Gibco, Life Technologies) according to the manufacturer and previous protocols[25,26]. The cells were stained with Oil Red O for visualization of fat droplets (Sigma-Aldrich), with Alizarin Red S for the calcium deposition (Sigma-Aldrich), and with Alcian Blue 8GX (Sigma Aldrich) for proteoglycans, respectively. Images were taken by standard bright field microscopy (Olympus CKX41; Life Science Solutions, Ballerup, Denmark) to evaluate the differentiation capabilities.

Preparation of conditioned media

To produce ASC-conditioned medium (ASC-CM) for subsequent testing on a fibroblast model, immediate-thawed ASCs were seeded at 20.000 cells/cm^2 in T175 flasks and allowed 24 h for recovery from the freezing-

thawing process. Cells were re-passaged, grown until reaching 80% confluency, and replenished with fresh growth medium. After 24 h, the conditioned medium was harvested, centrifuged at 500 g for 10 min, filtered to remove dead cells/debris, and then stored at − 80 °C.

Proliferation assay

Proliferation assays were performed on ASCs and on fibroblasts. Cells were seeded at a density of 600 cells per cm^2 in 96-well plates (Greiner Bio-one) and cultured in growth media (ASC) or conditioned media (fibroblasts). At days 1, 3, 5,7, 9,11,13, and 15, 5 replicate wells were lysed using 0.02% SDS (Sigma-Aldrich), and DsDNA content was quantified by a QUAN-IT PicoGreen kit (Thermo Fisher Scientific) following the manufacturer's instructions. Fluorescence was measured at 485/535 nm using an EnSpire Multimode Plate Reader (PerkinElmer, Boston, MA, USA).

Fibroblast migration assay

For each donor, human dermal fibroblasts (HDFs, Life Technologies, Frederick, MD, USA) were seeded into 12 replicate wells in a 96-well plate, grown until reaching full confluency, whereafter a scratch was made using an Autoscratcher (Agilent, Glostrup, Denmark). The medium was then replaced with ASC-conditioned medium. To monitor the scratch closure, the plate was placed into a standard incubator with a time-lapse microscope (Omni, CytoSmart, Eindhoven, Netherlands). Brightfield images were taken every 4 h for 68 h. The quantification of the scratch area was performed using a scratch assay algorithm provided by the microscope manufacturer.

Statistical test

All data are presented as mean + SEM. A student's t-test was employed to compare the two groups for the CFU assay. A two-way repeated measures ANOVA with a LSD test was conducted for the phenotype, proliferation, and scratch assay. A p value < 0.05 was considered statistically significant. Statistical analysis was conducted by SPSS version 27.0 (SPSS, Chicago, IL).

Data availability

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request. Data are located in controlled access data storage at Aalborg University.

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Author contributions

Conceptualization, Q.P.; methodology, G.R., M.B.S. and Q.P.; formal analysis, G.R., M.B.S. and Q.P.; investigation, Q.P., G.R. and V.Z.; resources, S.R.P. and T.F.; data analysis, Q.P., G.R. H.A., F.D., and V.Z.; writing - original draft preparation, G.R. and Q.P.; and writing—review and editing, S.R.P., T.F. and V.Z.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to Q.P.

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