Molecular Therapy
Oncology
Original Article

Optimized protocol for culturing menstrual blood-derived MSCs for combination with oncolytic adenoviruses in cancer treatment

Marcel Costa-Garcia,[1] Laura Moya-Borrego,[1] Ramon Alemany Bonastre,[1] and Rafael Moreno Olié[1]

[1]Cancer Immunotherapy Group, Oncobell and iProCURE programs, IDIBELL-Institut Català d'Oncologia, 08907 L'Hospitalet de Llobregat, Barcelona, Spain

Oncolytic viruses (OVs) are a promising therapeutic approach for cancer, although their systemic administration faces significant challenges. Mesenchymal stem cells have emerged as potential carriers to overcome these obstacles due to their tumor-tropic properties. This study investigates the use of menstrual blood-derived mesenchymal stem cells (MenSCs) as carriers for OVs in cancer therapy, focusing on enhancing their efficacy through different culture conditions. MenSCs were isolated from donors of different ages and cultured under normoxic and hypoxic conditions, with varying adherence capacities. Hypoxic conditions significantly improved MenSCs proliferation and tumor migration capabilities, as demonstrated by proliferation assays and RNA-sequencing analysis, which revealed upregulation of genes related to cell division and tumor tropism. *In vivo* studies using a lung adenocarcinoma mouse model confirmed that hypoxia-conditioned MenSCs had superior tumor-homing abilities. The study also demonstrated the feasibility of establishing a master and working cell bank from a single menstrual blood donation. These findings suggest that hypoxia-conditioned MenSCs could be highly effective as OV carriers, potentially leading to better clinical outcomes in cancer treatment by enhancing tumor targeting and therapeutic efficacy.

INTRODUCTION

The use of oncolytic viruses (OVs) for cancer treatment has significantly increased over the past 20 years, with numerous candidates entering clinical trials and three receiving approval for specific indications.[1] However, OVs face multiple challenges that hinder their effective application, particularly when administered systemically. These challenges include liver sequestration, neutralizing interactions in the blood, physical barriers to infection, and rapid clearance by the immune system.[2] Several strategies are being developed in order to overcome these obstacles (reviewed in Shalhout et al.[3,4]). One promising approach is the use of cell carriers for systemic delivery of OVs to primary tumors and metastases. Cell carriers can protect OVs from complement or neutralizing antibodies, evade filtering organs, and cross the endothelial barrier due to their ability to migrate to different tissues and organs following chemokine gradients or using cell surface adhesion proteins.

Mesenchymal stem cells (MSCs) are particularly appealing as cell carriers for OVs due to their ease of isolation and expansion, immune evasion properties, and ability to migrate to tumors. ALO/CELYVIR, an advanced therapy medicinal product (ATMP) comprising bone marrow-derived mesenchymal stem cells (BM-MSCs) carrying the oncolytic adenovirus (OAd) ICOVIR-5 (developed in our laboratory[5]) has completed two clinical trials and is currently being evaluated in three ongoing trials. Clinical results have shown an absence of toxicity or disease progression related to the infusion of ALO/CELYVIR, and a modest clinical outcome, with two complete responses and 12 responses (partial or stabilized disease) among the 65 patients treated in the different trials. Among other limitations, we identified two main drawbacks that limit the effectiveness of this therapy: the poor arrival of cells to the tumor and the heterogeneity of the cellular product. Concerning the first issue, it has not been possible to determine the arrival of infected mesenchymal cells to the tumor. Regarding the impact of cellular heterogeneity on the efficacy of ALO/CELYVIR, a comparative study between patients who responded to ALO/CELYVIR vs. non-responders revealed that the properties of the mesenchymal cells, in terms of expression levels of adhesion molecules, migration capacity, and immune-related molecules, influenced treatment outcomes.[6]

Over the past years, our group has focused on improving ALO/CELYVIR's clinical outcomes by optimizing both the viral and cellular components of the therapy. Initially, we validated and demonstrated that menstrual blood should be considered an optimal source for developing "off-the-shelf" ALO/CELYVIR therapies as an alternative to more classical sources of MSCs, such as bone marrow or adipose tissue.[7,8] In this follow-up study, we aimed to assess the influence of various parameters on the proliferation potential and protein expression profile of menstrual blood-derived mesenchymal stem cells (MenSCs) related to their ability to migrate to tumors and regulate the immune system. First, we examined donor age, as it has been previously reported that MenSCs exhibit a downregulation of genes involved in cell proliferation and migration with increasing donor

Received 1 August 2024; accepted 14 November 2024;
https://doi.org/10.1016/j.omton.2024.200907.

Correspondence: Rafael Moreno Olié, Cancer Immunotherapy Group, Oncobell and iProCURE programs, IDIBELL-Institut Català d'Oncologia, 08907 L'Hospitalet Llobregat, Barcelona, Spain.
E-mail: rafamoreno@iconcologia.net

age.[9] Second, we investigated oxygen culture conditions, as it has been shown that MSCs cultured under hypoxia have an increased ability to migrate toward tumors.[10] Finally, we evaluated *in vitro* plastic adhesion capacities, since Bolontrade et al. described a highly adherent MSC subpopulation with enhanced tumor-homing capacity.[11] Additionally, we determined the changes in gene expression levels that occur in MenSCs following viral infection to assess how these changes might affect the efficacy of cell therapy.

Our results have allowed us to establish an optimized protocol to generate a master MenSC cell bank, enabling the design of a future ALO/CELYVIR version 2 that combines mesenchymal cells with enhanced tumor migration capacity and oncolytic adenoviruses.

RESULTS

Donor and sample characterization

In social terms, several women expressed interest in donating menstrual blood for the purpose of obtaining mesenchymal cells after being informed about the project objectives. Out of the 19 women who initially showed interest, 16 ultimately donated menstrual blood for the project, resulting in an 86% success rate. Notably, 100% of the women in the 25–35 and >35 age groups donated blood (5 of 5 and 6 of 6, respectively), while only 66.6% of the women in the 18–25 age group ultimately provided a sample (5 of 8).

Regarding the menstrual blood samples, a total of 19 samples were received from 16 donors, distributed as follows: seven samples from the 18–25 years group, five samples from the 26–35 years group, and seven samples from the >35 years group (Table S1 details the main characteristics of each sample). Of the 19 samples received, mesenchymal stem cell (MenSC) populations were successfully established in 15 of them (79%). One sample from a donor in the >35 group exhibited fungal contamination a few days after the cultures were initiated; however, a subsequent donation from the same donor successfully established the cell population. Additionally, three samples from three consecutive cycles from a donor in the 18–25 age group showed fungal contamination within days of culture, possibly due to a subclinical infection.

The process of menstrual blood donation, purification, isolation, and amplification of MenSC populations is summarized in Figure 1A. Once amplified at passage 3, colony-forming unit assays and gene expression analysis by RNA sequencing (RNA-seq) were performed. Figure 1B summarizes the different populations established for each sample and the analyses performed.

MenSCs growth potential

We evaluated the capability of individual cells from each culture to growth after 10 days in culture. As observed in Figure 2, regardless of their adherence capacity to plastic, cells cultured under hypoxic conditions exhibited a higher proliferation capability compared with those cultured under normoxic conditions. These differences were statistically significant for the 18–25 and the 26–35 years age groups. No differences were observed between samples with high or low adherence cultured under the same O_2 conditions.

MenSCs expression profile

In an initial analysis, we aimed to evaluate the influence of the three variables characterizing our cellular populations (donor age, adherence capacity, and O_2 condition), on three cellular parameters of special interest in our project: the expression of chemokine receptors, proliferation capacity (nuclear division), and tumor tropism. For the first two parameters, gene expression signatures were constructed based on gene sets obtained from the MSigDB[12]; for tumor tropism, all receptors described with implications in tumor homing for mesenchymal stem cells were studied[13] (the list of all genes analyzed for each parameter is detailed in Table S2). Total RNA from all four culture conditions from 13 donors (three from 18–25 years, five from 25–35 years, five from >35 years) were extracted and analyzed. As observed in Figure 3A, the only variable that has a significant influence on those selected cellular properties is the oxygen concentration during cell culture.

Following this observation, we decided to compare populations exclusively based on oxygen concentration during expansion. First, we compared the differential gene expression from cells cultured under hypoxic vs. normoxic conditions. As shown in Figure 3B, a substantial number of genes were either upregulated (right, blue) or downregulated (left, red) under hypoxia (the complete list of upregulated and downregulated genes is indicated in Table S3). Cellular functions enriched or reduced under hypoxia were estimated performing a gene ontology (GO) enrichment analysis (Figure 3C; Table S4). While the functions downregulated under hypoxia were primarily related to cellular metabolism and biosynthesis of compounds, those enriched under hypoxia are more associated with hypoxia response pathways, including glycolysis, as well as cell growth and tissue migration. As expected, a subsequent heatmap analysis revealed an upregulation of numerous genes involved in cell proliferation and tumor migration processes (Figure 3D).

Finally, we aimed to determine if there was a correlation between RNA-seq nuclear division score from enriched genes with culture measures of occupied area. As depicted in Figure 3E, both growth potential data (% occupied area) and RNA-seq showed a clear positive correlation, and samples cultured under hypoxia and normoxia were clearly separated, the former showing higher colony-forming unit (CFU) and Nuclear Division score.

MenSCs tumor migration *in vivo*

To evaluate the impact of gene overexpression related to MenSCs tumor migration cultured under hypoxic conditions within an animal model, tumors derived from the A549 human lung adenocarcinoma cell line were implanted in NOD scid gamma (NSG) immunodeficient mice. Upon reaching the appropriate tumor size, MenSCs from the same donor, cultured simultaneously under hypoxic or normoxic conditions, were genetically modified with a lentivirus to stably express the luciferase gene and were administered systemically to the

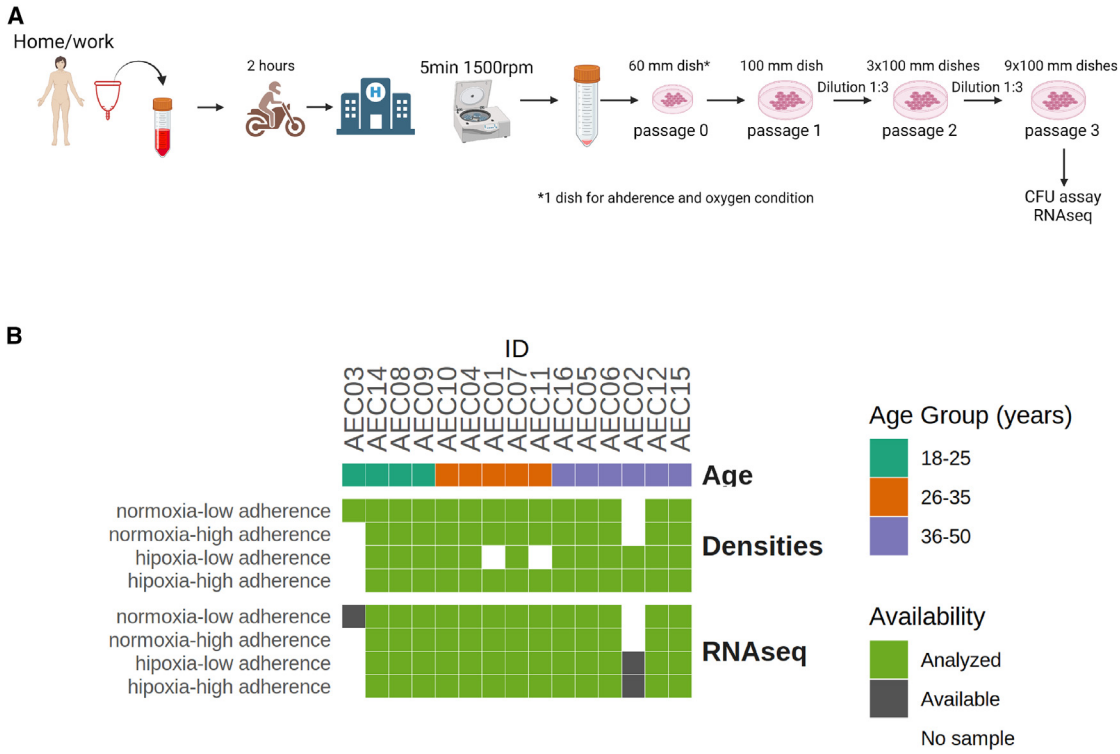

Figure 1. Summary of the process for obtaining and amplifying different populations of mesenchymal cells and the analyses performed

(A) Menstrual blood is collected by the donor at home or work and sent via local courier to the laboratory within 2 h. After washing with PBS and centrifugation, the four cell populations resulting from the combination of oxygen concentration and adherence (see materials and methods) are amplified through a 1:3 dilution (created with BioRender.com). (B) Summary of the different cell populations obtained from 15 menstrual blood donations, organized by donor age groups. The analyses performed for each cell population are also indicated.

mice (2×10^6 cells per mouse). Using an *in vivo* imaging system, the number of cells migrating to the tumor was quantified daily over time. As illustrated in Figure 4, MenSCs cultured under hypoxic conditions demonstrated a significantly enhanced capacity for migration (Figures 4A and S1) and accumulation (Figure 4B) within the tumor compared with those cultured under normoxic conditions, corroborating the RNA-seq analysis findings.

MenSCs expansion potential

With the ultimate goal of determining whether MenSCs cultured under hypoxic conditions could give rise to a potential master cell bank and working bank for the reformulation of ALO/CELYVIR, we conducted an experiment to quantify the total number of cells that could be obtained from a single donor within a single menstrual cycle. This was done to assess whether the required numbers of MSCs for a clinical trial could be achieved. Blood was collected from the same donor during the first 3 days of the cycle (across three different cycles) and subjected to three different processing methods: processing the blood immediately after donation (test 1), leaving the blood at room temperature for 24–48 h before processing (test 2), and collecting multiple times each day with each sample left at room temperature for 24 h before processing (test 3) (Figure 5A). As observed in Figure 5B, irrespective of the processing method followed, after 34 days of culture under hypoxic conditions (seven passages), we successfully amplified the MenSCs to obtain more than 10^9 cells. This result confirms the robustness of the methodology for obtaining and amplifying MenSCs.

Viral and cellular gene expression from infected MenSCs

Since many adenoviral genes are designed to redirect the cellular gene expression machinery, promoting viral replication while compromising cellular functions, we wanted to analyze in parallel the kinetics of viral and cellular gene expression to understand how viral infection affects cellular functions.

MenSCs cultured under hypoxic conditions were infected with the oncolytic adenovirus ICOVIR15, and RNA was extracted and analyzed at various time points post-infection to compare the kinetics of viral and cellular gene expression.

In an initial analysis, we observed that the virus is capable of hijacking the expression machinery of infected cells to favor the expression of viral genes. As shown in Figure 6A, there is a decrease in the expression of cellular genes over time post-infection, following a kinetics pattern inverse to that of viral gene expression (Figure 6B). Focusing on cellular genes, we identified three main metaclusters based on their expression kinetics: genes whose expression is downregulated

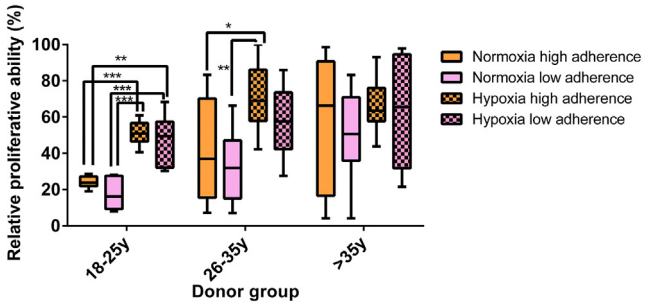

Figure 2. Relative proliferation ability of individual cells after 10 days in culture

The y axis surface occupied after culture. The x axis age donor groups. The relative proliferation ability for each population of mesenchymal cells is represented by calculating the percentage of occupied culture area (y axis) as a function of the donor's age (x axis). Comparison of the cell growth potential based on oxygen concentration and adherence for each analyzed donor age group is indicated. Bars, mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

immediately after viral infection (m1), genes that remain stable during the first 24 h post-infection and then downregulated (m2), and genes that are upregulated during the first 24 h of infection and then downregulated (m3) (Figure 6C). Performing a GO enrichment analysis for each metacluster allowed us to determine the main processes affected by these kinetics (Figure 6D; Table S5). Metacluster 1 (m1) showed an enrichment for cytoskeleton and organelle reorganization. Metacluster 2 (m2) was enriched in genes regulating RNA biosynthesis and modification, while the most enriched processes in metacluster 3 (m3) included DNA replication in addition to the RNA-related processes observed in m2. The different kinetics in the regulation of these processes may reflect the modulation of cellular pathways by the viral cycle. We were also interested in the kinetics of genes related to the "tissue migration" and "cell growth" gene ontologies, as previously highlighted (Figures 3C and 3D), as these functions are essential to the efficacy of the proposed therapy. We found that both ontologies were significantly enriched in the m1 metacluster, indicating that genes associated with these functions were downregulated immediately after viral infection.

DISCUSSION

Menstrual blood has proven to be an interesting alternative source for obtaining MSCs, compared with classical sources such as bone marrow or adipose tissue, with clear advantages over the latter, including the ability to allow easy isolation without clinical intervention or hospitalization, and enabling repeated and periodic donations.[14] Additionally, MSCs derived from menstrual blood have been shown to share the same immunoprivileged profile and immunoregulatory capacity as those obtained from other sources, with a greater and faster proliferation capacity *in vitro*.[15] During the last years, our group has sought to harness these advantages by using MenSCs as cellular carriers for our oncolytic adenoviruses, and we have demonstrated that these cells are not only capable of delivering and releasing our viruses into the tumor, but also of stimulating the immune system to recognize and eliminate the tumor.[8]

In this work, we address critical limitations identified in previous clinical trials evaluating the combination of mesenchymal stem cells and oncolytic adenovirus for cancer treatment, such as the suboptimal delivery of mesenchymal stem cells to tumor sites and the heterogeneity of the cellular product.[16] The findings of this study underscore the potential of menstrual blood-derived mesenchymal stem cells (MenSCs) as promising cell carriers for oncolytic viruses (OVs) in cancer therapy. Specifically, the enhanced tumor migration and proliferation capabilities of MenSCs cultured under hypoxic conditions could significantly improve the efficacy not only of ALO/CELYVIR therapy, but of any antitumor therapy that requires MSCs as a cellular vehicle for intratumoral delivery of the therapeutic agent.[17]

We have highlighted that, in terms of donor recruitment and sample handling (with samples collected by the donors themselves), as well as ethical considerations, menstrual blood is far superior to other sources of mesenchymal cells that require donor assistance at a hospital and a more or less complex surgical intervention.[18] Thus, this study emphasizes the highly favorable attitude of women toward donating menstrual blood, which is in line with previous studies.[19] Additionally, as previously reported, among our donors, the age group of 18–25 was slightly less inclined to donate blood samples, likely because healthcare is not a priority for this age group, as postulated by Manley and coworkers.

The inherent tumor migration capacity of MSCs[20] can be enhanced under certain circumstances. For instance, low oxygen concentrations during the expansion of mesenchymal cells increase their ability to migrate toward tumors through the upregulation of chemokine receptors.[10] Additionally, Bolontrade et al. described a specific MSC subpopulation, characterized by rapid adherence to the culture substrate, that exhibited higher tumor migration.[11] In this study, we aimed to determine whether these two factors, the oxygen concentration during cell expansion and the high adherence capacity, along with the age of the donors, could influence the cell proliferation potential and tumor migration capacity of our MenSCs. To this end, we conducted assays on both cell proliferation and gene expression. Our results demonstrated that MenSCs cultured under hypoxia showed enhanced colony-forming unit (CFU) capabilities. Moreover, the RNA-seq analysis further supported these findings, highlighting significant upregulation of genes associated with cell proliferation and tumor migration under hypoxic conditions. As expected, among the functions with the highest fold-change in hypoxia compared with normoxia are those that respond to an oxygen reduction. It is well established that decreased oxygen concentration stimulates different pathways, most of them controlled by the regulated expression and function of the hypoxia-inducible transcription factor (HIF) family proteins, which has been described to play a role in enhancing cellular migration and invasion capacity, among many other functions.[21,22] Thus, it is plausible to postulate that the tumor-homing-related functions increase observed under hypoxia, such as leukocyte chemotaxis and migration, regulation of cell adhesion mediated by integrins, and tissue migration, is regulated by the response of HIF target genes to decreased O_2 levels. This was consistent across various donor ages and adherence capacities,

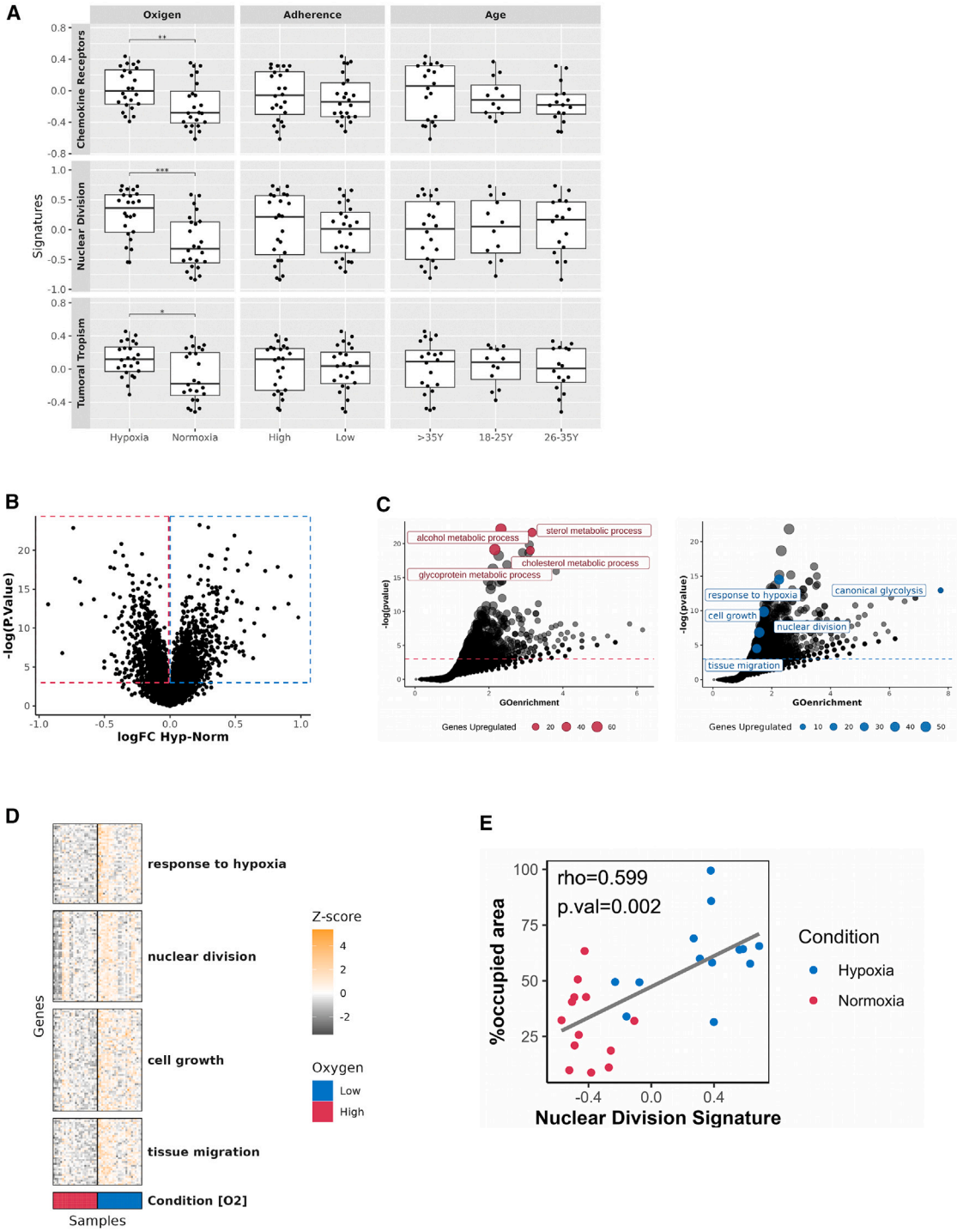

Figure 3. Gene expression modulation under hypoxia

(A) RNA-seq expression in MenSCs, comparing different variables including oxygen concentration, adherence to plastic and donor age. Scatterplot represents GSVA score for "Chemokine Receptors," "Nuclear Division," and "Tumoral Tropism" signatures comparing different categories of each variable. Boxplots represent values from the first to the third quartile. The median is also represented. *$p < 0.05$; **$p < 0.01$. (B) Volcano plot representing differential expression of genes under hypoxia (right) or normoxia (left) conditions. Blue and red squares identify genes with positive (enriched under hypoxia) or negative (diminished under hypoxia) fold changes and significant p values,

(legend continued on next page)

suggesting that hypoxia is a pivotal factor in optimizing MenSC functionality. Neither age nor high adherence to the culture plastic showed an influence on the proliferation and tumor migration capacity of MenSCs. While the influence of age was previously unknown and investigated for the first time in this study, we were surprised to find no differences between cells with high and low adherence, at least in terms of the expression of genes related to tumor migration, as previously described. A possible explanation could be that after separating the cells based on differential adhesive behavior during the first hour of culture, the cells were cultured under identical conditions for about 30 days until passage 3, when the assays were conducted, potentially mitigating the initial differences between cell populations over time.

In a previous study, we determined that following intraperitoneal administration, MenSCs had the ability to migrate to various tissues such as the liver, spleen, and lungs, although they exhibited a greater propensity to migrate toward tumors.[7] In the present work, our *in vivo* experiments corroborated the *in vitro* findings, showing that hypoxia-conditioned MenSCs had a markedly higher tumor-homing capacity compared with their normoxia-cultured counterparts. The use of a bioluminescent imaging system allowed for quantification of cell migration, illustrating the potential for these cells to effectively target and accumulate in tumor tissues. This enhancement in tumor targeting is crucial for the efficacy of cell-based OV therapies, as it ensures a higher concentration of therapeutic agents at the tumor site, potentially leading to better clinical outcomes.

Based on these results, we identify MenSCs cultured under hypoxic conditions (irrespective of donor age and initial plastic adherence) as those presenting the highest growth potential and sustained expression of tumor migration molecules and, therefore, candidates for the development of the new ALO/CELYVIR therapy.

The successful amplification of MenSCs from a single donor to clinically relevant numbers within 34 days under hypoxic conditions is particularly noteworthy. This finding not only confirms the robustness of our culture methodology but also demonstrates the feasibility of producing sufficient cell quantities for extensive clinical trials from a single menstrual cycle. In this regard, ALO/CELVIR is being administered to patients at a dosage of 0.5×10^5 cells/kg, with six doses per patient.[16] Our results indicate that potentially from a single menstrual cycle, up to 1×10^9 MenSCs can be obtained, which would amount to 2,000 doses, sufficient to treat more than 300 patients. The ability to generate a master MenSCs cell bank ensures a consistent and scalable source of therapeutic cells, addressing one of the primary logistical challenges in developing "off-the-shelf" cell therapies.[23]

Although numerous studies have analyzed the capacity of MSCs to produce not only oncolytic adenoviruses but also other types of OVs,[13] to our knowledge, the kinetics of adenoviral and cellular gene expression during the infection process and the release of new viral progeny have never been analyzed. Moreover, considering that our therapy involves using mesenchymal cells to deliver and release oncolytic adenoviruses into the tumor, and that the time window for this function is 72–96 h (the time required for the oncolytic adenovirus to complete its replicative cycle), we believe it is crucial to determine how the kinetics of viral and cellular gene expression overlap. This understanding is essential for optimizing the infection process without affecting the tumor migration and the release of the maximum number of viruses within the tumor. In this regard, we observed a general downregulation of cellular genes, consistent with previous reports in other cell types.[24–26] We were able to identify several kinetic patterns that suggest a tuned regulation of this downregulation by the adenovirus along the viral cycle. A main concern arising from this observation is that processes related to tissue migration and cellular division seem to be downregulated very early upon viral infection. In this context, reducing the time between the mesenchymal cell infection process and the administration of the therapy to patients could positively impact the migration of mesenchymal cells to the tumor and, consequently, the therapeutic efficacy of the treatment. Currently, to generate ALO/CELYVIR, the infection process lasts for 2 h, after which the cells are infused into the patient. Our results support the appropriateness of this part of the procedure in the treatment with ALO/CELYVIR.

In conclusion, our study highlights the potential of hypoxia-conditioned MenSCs as enhanced cell carriers for oncolytic virotherapy. The improved tumor-homing capabilities and proliferation potential of these cells, combined with the ability to generate large quantities from a single donor, represent significant advancements toward the development of more effective and scalable cancer treatments. Future studies should focus on further optimizing the interaction between MenSCs and various oncolytic viruses, as well as conducting clinical trials to validate these findings in human subjects.

These results establish a robust basis for the development of an optimized version of CELYVIR therapy, potentially improving therapeutic outcomes in future clinical trials. In this context, the use of allogeneic cells is always subject to the potential issue of immune rejection. However, three clinical trials are currently under way using ALO/CELYVIR (EudraCT2019-001154-26; EudraCT 2020-004838-37; NCT05047276), with over 20 patients treated, and no immune response against the allogeneic mesenchymal cells has been observed in any patient. Since MenSCs share immunoregulatory properties

respectively ($p < 0.05$). (C) Significantly higher or lower expressed genes in hypoxia were selected and submitted to a GO enrichment analysis to elucidate which biological processes were enriched and diminished. The dashed lines represent the p-value of 0.05. Some processes we were interested in are highlighted and nametagged in the plot. (D) Heatmap representation of the enriched genes from the biological processes highlighted in (C). (E) A GSVA signature score for each sample was calculated using the enriched genes from "nuclear division" GO and its correlation to the experimental occupied culture area is plotted. Samples cultured in hypoxia or normoxia are colored in blue and red, respectively. Spearman correlation was applied.

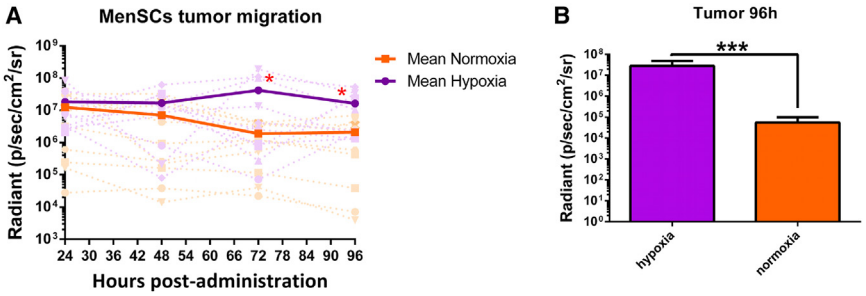

Figure 4. *In vivo* tumor migration capacity of MenSCs cultured under normoxia or hypoxia conditions

(A) Kinetics of tumor migration of MenSCs modified to express luciferase over time. Luminescence signal was detected using IVIS at the indicated time points after cell administration and quantified with IVIS software. The dark-colored lines represent the group mean, and the light-colored lines represent the individual animals. $^{*}p < 0.05$. (B) Presence of MenSCs at the end of the study. At 96 h post-administration, the animals were euthanized, and the tumors were excised and individually analyzed using IVIS. Bars, mean ± SEM. $^{***}p < 0.001$.

with BM-MSCs, and considering that the viral cycle ensures the elimination of the cells within 48–72 h post-administration, we do not anticipate an issue of rejection against MenSCs in a potential clinical trial.

Efforts will be crucial in translating the promising preclinical results into tangible clinical benefits for cancer patients. In this regard, our group has initiated a project focused on generating a master cell bank and a working cell bank of MenSCs produced under good manufacturing practices conditions, in collaboration with the Advanced Therapy Medicine Production Unit responsible for the current ALO/CELYVIR production. The ultimate goal is to submit an investigational medicinal product dossier (IMPD) for the treatment of glioblastoma using these MenSCs in combination with our oncolytic adenoviruses. Throughout this process, we aim to identify potential clinical-scale limitations of the proposed therapy and assess its clinical potential.

MATERIALS AND METHODS

Cell culture

Isolation of human menstrual blood-derived mesenchymal stem cells

Menstrual blood (1–5 mL) was collected from healthy female donors ($n = 16$) during the first 3 days of the menstrual phase using a menstrual cup (Enna cycle, Spain). Donors were classified into three age groups: 18–25, 25–35, and >35 years old. The protocol and cell donation for research purposes was approved by the Ethics Committee for Research at the Bellvitge University Hospital, and written consent was obtained for each donor. Blood samples were transferred to a sterile 50-mL centrifuge tube with conical bottom, filled with sterile phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA), and centrifuged at 1,500 rpm for 5 min. The pellet was resuspended in α-MEM (Life Technologies) containing 20% fetal bovine serum, 1% penicillin/streptomycin, and 1% gentamicin/amphotericin B (all from Life Technologies), and seeded in 60-mm culture dishes (Corning Inc., Corning, NY) at 37°C in a 5% CO_2 atmosphere under normoxic or hypoxic (2% O_2) conditions in a Heracell VIOS 160i CO_2 incubator with O_2 control system. No reagent to lysate erythrocytes is used in this process, thus the initial number of MenSCs cannot be calculated. After 1 h in culture, non-adherent cells were transferred to new 60-mm culture dishes and cultured again under same oxygen conditions. Thus, four different cell cultures were obtained from each sample: high adherence and low adherence were cultured in normoxia and hypoxia conditions. The next day, the cell monolayer was washed thrice with PBS to remove non-adherent cells and cellular debris and fresh medium was added. MenSCs were amplified for 6–20 days (passage 0), with the culture medium replaced every 3–4 days until 75%–90% confluency, followed by periodic passaging at a one-third dilution using TrypLE Express (Life Technologies) after achieving a subconfluent monolayer.

For the potential MSCs master and work bank generation from menstrual blood, three different donation and culture settings were established for the same donor (29 years old) based on three different menstrual cycles within a period of 7 months. In the first experimental condition, blood samples were collected at four time points during the cycle (24, 30, 48, and 54 h), processed immediately after receipt, then placed in culture under hypoxic conditions without differentiating high or low adherence. In the second setting, blood samples were collected at different times during the first 3 days of the cycle. Blood samples from the same day were collected in the same tube and kept at room temperature for 24–48 h before processing and culturing as before. In the third setting, three independent blood samples were obtained for each of the first 3 days of the cycle, stored at room temperature for 24 h in independent tubes, and then processed and cultured as previously described. For each setting, cells were amplified up to 80%–90% confluency and passaged at a one-third dilution until passage seven (approximately 32–34 days). The cells were counted, and the total number of cells in culture in each passage was estimated.

Cell lines

A549 (human lung adenocarcinoma) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Invitrogen) at 37°C, 5% CO_2. Cells were routinely tested for mycoplasma presence.

CFU assay

MenSCs from all donors and all four culture conditions (passage three) were seeded at clonal density of 500 cells/cm^2 in six-well cell

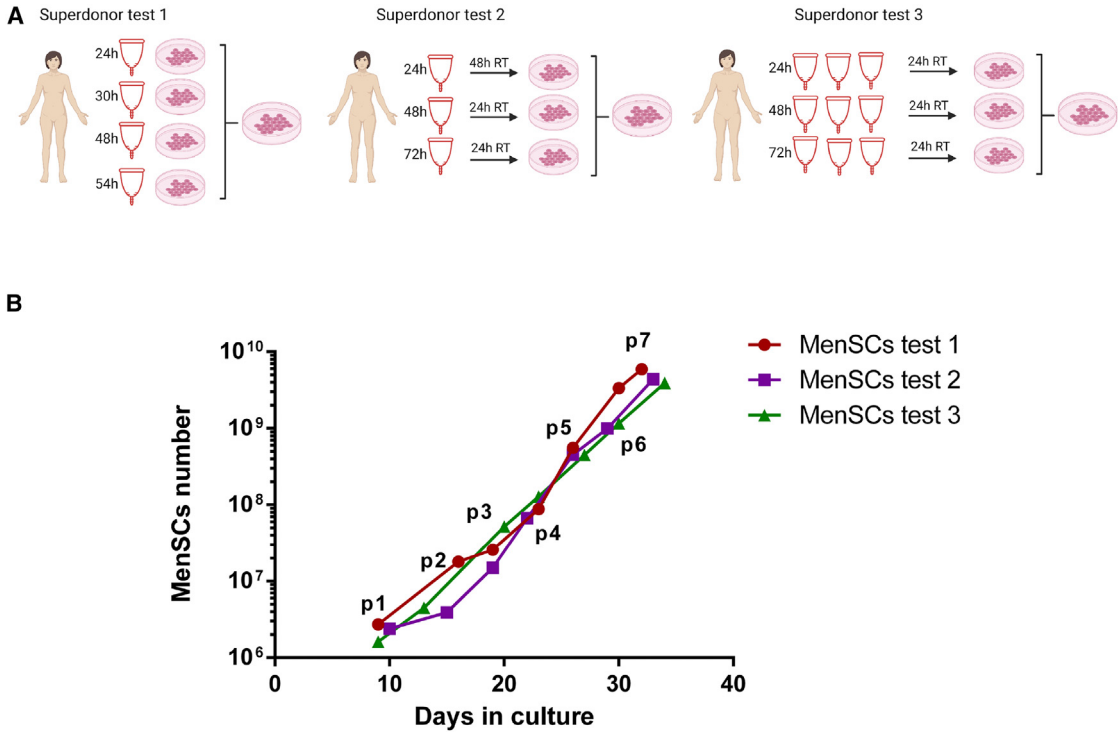

Figure 5. Estimation of the capacity for obtaining and expanding MenSCs from multiple donations by a single donor during one menstrual cycle

(A) Multiple menstrual blood donations within the same menstrual cycle were collected and processed as illustrated (with the same donor in all three experiments over a period of 7 months from the first to the last experiment) (created with BioRender.com). (B) The total number of MenSCs was calculated over 32 days of culture expansion. P1–P7 represent successive culture passages.

culture plates. After 10 days in culture, cells were washed with PBS, fixed with methanol for 10 min, and stained with 0.5% crystal violet for 20 min at room temperature. Plates were scanned with the Typhoon FLA 9500 biomolecular imager (GE Healthcare Life Sciences, Chicago, IL, USA) and the percentage of the well surface occupied by cells was calculated with ImageJ software.

RNA-seq library preparation, sequencing, and analysis

Total RNA was extracted from MenSCs (passage three) using RNeasy Mini Kit (QIAGEN, Valencia, CA). The integrity of the RNA was determined using the Qubit 4 fluorometer (Life Technologies). Library construction and sequencing was carried out by Macrogen using TruSeq Stranded Total RNA with Ribo-Zero Human kit and Novaseq 6000 device (150 cycles, 40M paired reads) (Illumina, Hayward, CA, USA). FASTQ files were mapped to the reference genome GRCh38/hg19 using the STAR aligner and quantified with RSEM. Counts were log-transformed and normalized with upper quantile prior to calculating the differential gene expression with "limma" R package. Signature scores were calculated using gene set variation analysis (GSVA) from "GSVA" R package and GO enrichment was calculated using the "clusterProfiler" R package. For "Cell Cycle" and "Chemokine Receptors" signatures, genes from gene sets "REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES" and "FISCHER_G2_M_CELL_CYCLE" from MSigDB were used.[12] For the "nuclear division signature," enriched genes in hypoxia from "nuclear division" GO were included.

For the cellular and viral gene expression kinetics experiment, 3×10^5 MenSCs (passage four) were plated in six-well plates and infected at a multiplicity of infection (MOI) of 500 ICOVIR15 (an oncolytic adenovirus designed in our laboratory[27]) per cell. At 24, 48, 72, and 96 h post-infection, RNA was extracted from the infected cells (as well as from non-infected control cells), processed, and analyzed as described above. To classify the cellular genes in unbiased clusters, the "hclust" R package was used, and they were further manually aggregated in several metaclusters. Genes included in the three main metaclusters were analyzed by GO enrichment using the "clusterProfiler" R package.

In vivo tumor-homing studies

In vivo studies were performed at the ICO-IDIBELL facility (Barcelona, Spain) AAALAC unit 1155, and approved by IDIBELL's Ethical Committee for Animal Experimentation.

Subcutaneous xenograft tumors were established by injecting 5×10^6 A549 cells (standard cell line used in efficacy and safety studies of oncolytic adenoviruses with MSCs[7,8,28]) into the flanks of 9-week-old NOD scid gamma (NSG) mice (to prevent immune reaction against

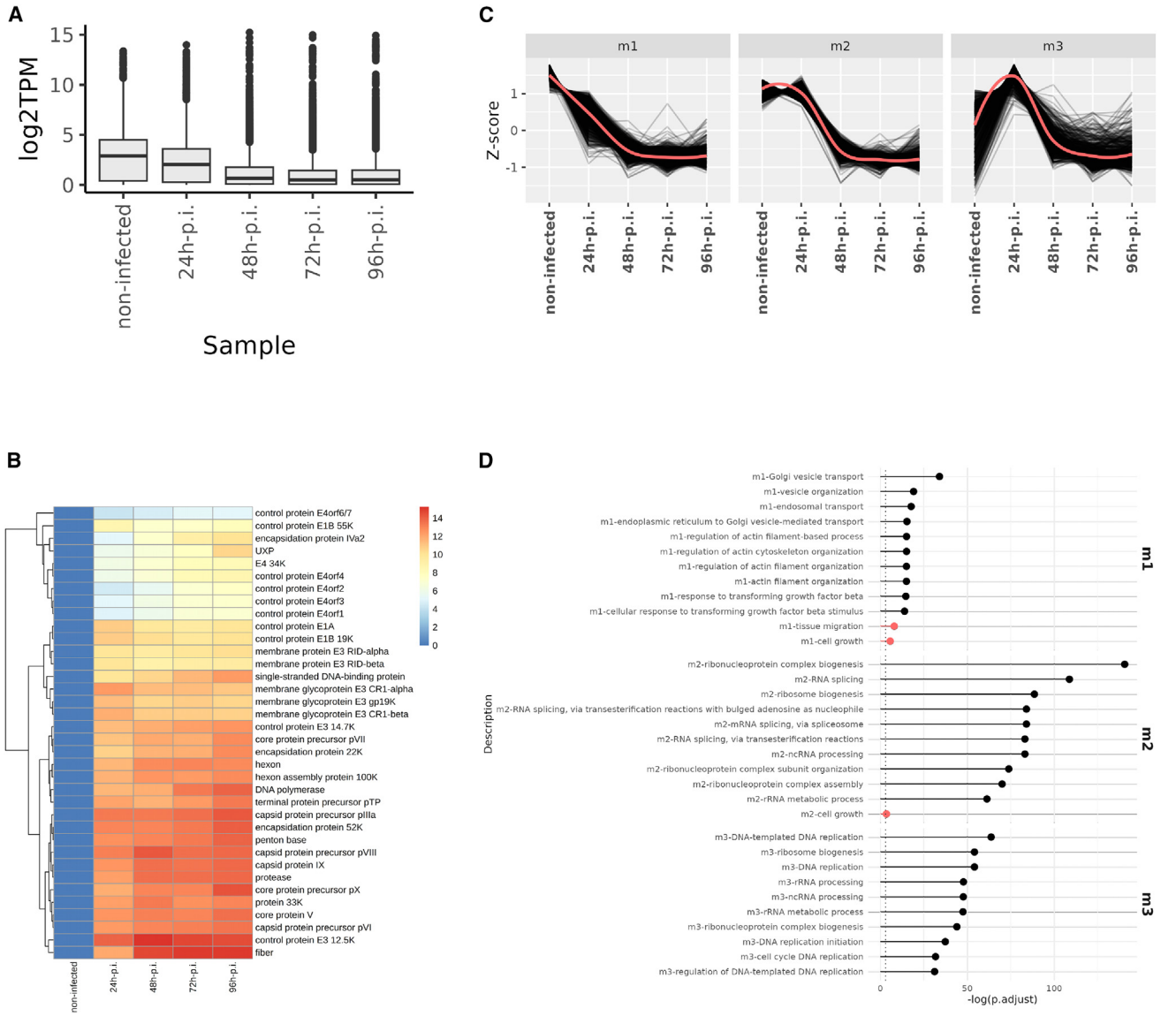

Figure 6. Kinetic expression of viral and cellular genes on MenSCs upon adenoviral infection

RNA-seq expression data was obtained from MenSCs after 24, 48, 72 and 96 h post-infection or non-infected as a control. (A) Boxplots showing Log2TPM cell genes expression levels across time points post-infection. Boxplots represent values from the first to the third quartile. The median is also represented. (B) Heatmap representing the expression levels of adenoviral genes over the time points. (C) Cellular genes were clustered based on their expression pattern throughout the viral infection and three main metaclusters were identified. Plots showing the kinetics of cellular genes stratified by those metaclusters. The red line summarizes the pattern using a local polynomial regression. (D) Genes from the three metaclusters were analyzed with GO enrichment and the top 10 enriched processes plus "tissue migration" and "cell growth" (in red) are plotted. Gray dotted line represents the significance limit (*p*.adjust of 0.05).

implanted human tumor cells and MenSCs). Simultaneously, MenSCs obtained from the same original blood sample and expanded in parallel under normoxia or hypoxia (passage four), were transduced with a lentiviral vector encoding GFP and luciferase (pTRPE-CBG-T2A-GFP, kindly provided by Dr. Sonia Guedan) and expanded (normoMenSCs-GL and hypoMenSCs-GL respectively, with transduction efficiency close to 85% in both case). When tumors reached 400 mm^3, animals were treated with a single intraperitoneal dose of 2×10^6 normoMenSCs-GL or hypoMenSCs-GL ($n = 5$).

To monitor MenSCs-GL tumor-homing, mice were intraperitoneally injected with 120 mg/kg body weight of firefly luciferin (Biosynth, Staad, Switzerland) at 24, 48, 72, and 96 h post-cell administration, and bioluminescent imaging analysis performed using the IVIS Lumina bioimaging system (PerkinElmer). Images were analyzed

with IVIS Living Image (PerkinElmer) software. Regions of interest (ROIs) were manually drawn around the tumors.

Statistical analysis

Statistical comparisons between two groups were performed using the Mann-Whitney *U* test. For comparison of more than two groups, the Kruskal-Wallis test with Dunn post hoc test was used. Statistical significance was established as $p < 0.05$. Data are presented as the mean ± SD or SEM. All statistical analyses were calculated with GraphPad Prism software and R.

DATA AND CODE AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ACKNOWLEDGMENTS

Special thanks to all sample donors for their selfless participation in the project. This work has been founded by IDEAS21912MORE grant to R.M.O. from Asociación Española Contra el Cáncer, PI23/00385 grant to R.M.O. from Instituto de Salud Carlos III, 2021SGR00895 research grant to R.A.B. and R.M.O. from the Generalitat de Catalunya, and the Spanish Network of Advanced Therapies (TeraV, https://www.redterav.es). TERAV Network is supported by Instituto de Salud Carlos III and funded by the European Union–NextGenerationEU, Recovery, Transformation and Resilience Plan (Reference RD21/0017/0032).

We thank CERCA Program/Generalitat de Catalunya for their institutional support.

AUTHOR CONTRIBUTIONS

R.A.B. and R.M.O. contributed to the study conception and design. Material preparation, and analysis were performed by R.M.O., M.C.-G., and L.M.-B. Experimental design and data collection were also performed by R.M.O. and M.C.-G. The manuscript was written by R.M.O. All authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

R.A.B. declares to be a part-time employee and stockholder of Theriva Biologics.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the author(s) used ChatGPT in order to improve language and readability of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2024.200907.

REFERENCES

1. Chen, L., Zuo, M., Zhou, Q., and Wang, Y. (2023). Oncolytic virotherapy in cancer treatment: challenges and optimization prospects. Front. Immunol. *14*, 1308890. https://doi.org/10.3389/fimmu.2023.1308890.

2. Zheng, M., Huang, J., Tong, A., and Yang, H. (2019). Oncolytic Viruses for Cancer Therapy: Barriers and Recent Advances. Mol. Ther. Oncolytics *15*, 234–247. https://doi.org/10.1016/j.omto.2019.10.007.

3. Shalhout, S.Z., Miller, D.M., Emerick, K.S., and Kaufman, H.L. (2023). Therapy with oncolytic viruses: progress and challenges. Nat. Rev. Clin. Oncol. *20*, 160–177. https://doi.org/10.1038/s41571-022-00719-w.

4. Harrington, K., Freeman, D.J., Kelly, B., Harper, J., and Soria, J.-C. (2019). Optimizing oncolytic virotherapy in cancer treatment. Nat. Rev. Drug Discov. *18*, 689–706. https://doi.org/10.1038/s41573-019-0029-0.

5. Cascallo, M., Alonso, M.M., Rojas, J.J., Perez-Gimenez, A., Fueyo, J., and Alemany, R. (2007). Systemic Toxicity–Efficacy Profile of ICOVIR-5, a Potent and Selective Oncolytic Adenovirus Based on the pRB Pathway. Mol. Ther. *15*, 1607–1615. https://doi.org/10.1038/sj.mt.6300239.

6. Melen, G.J., Franco-Luzón, L., Ruano, D., González-Murillo, Á., Alfranca, A., Casco, F., Lassaletta, Á., Alonso, M., Madero, L., Alemany, R., et al. (2016). Influence of carrier cells on the clinical outcome of children with neuroblastoma treated with high dose of oncolytic adenovirus delivered in mesenchymal stem cells. Cancer Lett. *371*, 161–170. https://doi.org/10.1016/j.canlet.2015.11.036.

7. Moreno, R., Rojas, L.A., Villellas, F.V., Soriano, V.C., García-Castro, J., Fajardo, C.A., and Alemany, R. (2017). Human Menstrual Blood-Derived Mesenchymal Stem Cells as Potential Cell Carriers for Oncolytic Adenovirus. Stem Cells Int. *2017*, 3615729. https://doi.org/10.1155/2017/3615729.

8. Moreno, R., Fajardo, C.A., Farrera-Sal, M., Perisé-Barrios, A.J., Morales-Molina, A., Al-Zaher, A.A., García-Castro, J., and Alemany, R. (2019). Enhanced Antitumor Efficacy of Oncolytic Adenovirus–loaded Menstrual Blood–derived Mesenchymal Stem Cells in Combination with Peripheral Blood Mononuclear Cells. Mol. Cancer Ther. *18*, 127–138. https://doi.org/10.1158/1535-7163.MCT-18-0431.

9. Chen, J., Du, X., Chen, Q., and Xiang, C. (2015). Effects of donors' age and passage number on the biological characteristics of menstrual blood-derived stem cells. Int. J. Clin. Exp. Pathol. *8*, 14584–14595.

10. Huang, Y.-F., Chen, M.-J., Wu, M.-H., and Hung, S.-C. (2013). The use of hypoxic cultured mesenchymal stem cell for oncolytic virus therapy. Cancer Gene Ther. *20*, 308–316. https://doi.org/10.1038/cgt.2013.22.

11. Bolontrade, M.F., Sganga, L., Piaggio, E., Viale, D.L., Sorrentino, M.A., Robinson, A., Sevlever, G., García, M.G., Mazzolini, G., and Podhajcer, O.L. (2012). A Specific Subpopulation of Mesenchymal Stromal Cell Carriers Overrides Melanoma Resistance to an Oncolytic Adenovirus. Stem Cells Dev. *21*, 2689–2702. https://doi.org/10.1089/scd.2011.0643.

12. Liberzon, A., Subramanian, A., Pinchback, R., Thorvaldsdóttir, H., Tamayo, P., and Mesirov, J.P. (2011). Molecular signatures database (MSigDB) 3.0. Bioinformatics *27*, 1739–1740. https://doi.org/10.1093/bioinformatics/btr260.

13. Moreno, R. (2021). Mesenchymal stem cells and oncolytic viruses: joining forces against cancer. J. Immunother. Cancer *9*, e001684. https://doi.org/10.1136/jitc-2020-001684.

14. Rossignoli, F., Caselli, A., Grisendi, G., Piccinno, S., Burns, J.S., Murgia, A., Veronesi, E., Loschi, P., Masini, C., Conte, P., et al. (2013). Isolation, Characterization, and Transduction of Endometrial Decidual Tissue Multipotent Mesenchymal Stromal/Stem Cells from Menstrual Blood. BioMed Res. Int. *2013*, 901821. https://doi.org/10.1155/2013/901821.

15. Darzi, S., Werkmeister, J.A., Deane, J.A., and Gargett, C.E. (2016). Identification and Characterization of Human Endometrial Mesenchymal Stem/Stromal Cells and Their Potential for Cellular Therapy. Stem Cells Transl. Med. *5*, 1127–1132. https://doi.org/10.5966/sctm.2015-0190.

16. Ruano, D., López-Martín, J.A., Moreno, L., Lassaletta, Á., Bautista, F., Andión, M., Hernández, C., González-Murillo, Á., Melen, G., Alemany, R., et al. (2020). First-in-Human, First-in-Child Trial of Autologous MSCs Carrying the Oncolytic Virus Icovir-5 in Patients with Advanced Tumors. Mol. Ther. *28*, 1033–1042. https://doi.org/10.1016/j.ymthe.2020.01.019.

17. Zhang, T., Lin, R., Wu, H., Jiang, X., and Gao, J. (2022). Mesenchymal stem cells: A living carrier for active tumor-targeted delivery. Adv. Drug Deliv. Rev. *185*, 114300. https://doi.org/10.1016/j.addr.2022.114300.

18. Jin, H.J., Bae, Y.K., Kim, M., Kwon, S.-J., Jeon, H.B., Choi, S.J., Kim, S.W., Yang, Y.S., Oh, W., and Chang, J.W. (2013). Comparative Analysis of Human Mesenchymal Stem Cells from Bone Marrow, Adipose Tissue, and Umbilical Cord Blood as Sources of Cell Therapy. Int. J. Mol. Sci. *14*, 17986–18001. https://doi.org/10.3390/ijms140917986.

19. Manley, H., Sprinks, J., and Breedon, P. (2019). Menstrual Blood-Derived Mesenchymal Stem Cells: Women's Attitudes, Willingness, and Barriers to Donation of Menstrual Blood. J. Womens Health *28*, 1688–1697. https://doi.org/10.1089/jwh.2019.7745.

20. Reagan, M.R., and Kaplan, D.L. (2011). Concise Review: Mesenchymal Stem Cell Tumor-Homing: Detection Methods in Disease Model Systems. Stem Cell. *29*, 920–927. https://doi.org/10.1002/stem.645.

21. Luo, Z., Tian, M., Yang, G., Tan, Q., Chen, Y., Li, G., Zhang, Q., Li, Y., Wan, P., and Wu, J. (2022). Hypoxia signaling in human health and diseases: implications and prospects for therapeutics. Sig. Transduct. Targeted Ther. *7*, 218. https://doi.org/10.1038/s41392-022-01080-1.

22. Bruick, R.K. (2003). Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. Genes Dev. *17*, 2614–2623. https://doi.org/10.1101/gad.1145503.

23. Mody, H., Sutaria, D.S., and Miles, D. (2024). Clinical Pharmacology Considerations for the "Off-the-Shelf" Allogeneic Cell Therapies. Clin. Pharmacol. Ther. *115*, 1233–1250. https://doi.org/10.1002/cpt.3241.

24. Zhao, H., Granberg, F., and Pettersson, U. (2007). How adenovirus strives to control cellular gene expression. Virology *363*, 357–375. https://doi.org/10.1016/j.virol.2007.02.013.

25. Ben-Israel, H., and Kleinberger, T. (2002). Adenovirus and cell cycle control. Front Biosci. *7*, 1369–1395. https://doi.org/10.2741/ben.

26. Ornelles, D.A., Gooding, L.R., Dickherber, M.L., Policard, M., and Garnett-Benson, C. (2016). Limited but durable changes to cellular gene expression in a model of latent adenovirus infection are reflected in childhood leukemic cell lines. Virology *494*, 67–77. https://doi.org/10.1016/j.virol.2016.03.015.

27. Rojas, J.J., Guedan, S., Searle, P.F., Martinez-Quintanilla, J., Gil-Hoyos, R., Alcayaga-Miranda, F., Cascallo, M., and Alemany, R. (2010). Minimal RB-responsive E1A Promoter Modification to Attain Potency, Selectivity, and Transgene-arming Capacity in Oncolytic Adenoviruses. Mol. Ther. *18*, 1960–1971. https://doi.org/10.1038/mt.2010.173.

28. Barlabé, P., Sostoa, J.d., Fajardo, C.A., Alemany, R., and Moreno, R. (2020). Enhanced antitumor efficacy of an oncolytic adenovirus armed with an EGFR-targeted BiTE using menstrual blood-derived mesenchymal stem cells as carriers. Cancer Gene Ther. *27*, 383–388. https://doi.org/10.1038/s41417-019-0110-1.