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# Facile bead-to-bead cell-transfer method for serial subculture and large-scale expansion of human mesenchymal stem cells in bioreactors

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## Abstract

The conventional planar culture of adherent cells is inefficient for large-scale manufacturing of cell and gene therapy products. We developed a facile and efficient bead-to-bead cell-transfer method for serial subculture and large-scale expansion of human mesenchymal stem cells (hMSCs) with microcarriers in bioreactors. We first compared culture medium with and without nucleosides and found the former maintained the expression of surface markers of hMSCs during their prolonged culture and enabled faster cell proliferation. Subsequently, we developed our bead-to-bead cell transfer method to subculture hMSCs and found that intermittent agitation after adding fresh microcarriers to cell-populated microcarriers could promote spontaneous cell migration to fresh microcarriers, reduce microcarrier aggregation, and improve cell yield. This method enabled serial subculture of hMSCs in spinner flasks from passage 4 to passage 9 without using proteolytic enzymes, which showed faster cell proliferation than the serial planar cultures undergoing multiple enzyme treatment. Finally, we used the medium containing nucleosides and our bead-to-bead cell transfer method for cell culture scale-up from 4- to 50-L cultures in single-use bioreactors. We achieved a 242-fold increase in the number of cells to $1.45 \times 10^{10}$ after 27-day culture and found that the cells harvested from the bioreactors maintained proliferation ability, expression of their surface markers, tri-lineage differentiation potential and immunomodulatory property. This study shows the promotive effect of nucleosides on hMSC expansion and the potential of using our bead-to-bead transfer method for larger-scale manufacturing of hMSCs for cell therapy.

**KEYWORDS**
bead-to-bead transfer, bioreactor, mesenchymal stem cell, microcarrier, nucleosides, scale up

## Significance statement

Large-scale expansion of human mesenchymal stem cells (hMSCs) in stirred tank bioreactors is challenging because hMSCs have limited proliferation potential and are sensitive to damage from enzyme treatment. The authors first discovered that a culture medium with nucleosides significantly promoted the proliferation of hMSCs in 2D and microcarrier-based suspension

culture. Subsequently the authors developed a bead-to-bead cell transfer process with intermittent agitation for cell culture scale-up in bioreactors, which maintained the proliferation, viability, and normal phenotype of hMSCs. The bead-to-bead cell transfer has great potential for large-scale manufacturing of hMSCs for cell therapy.

## 1 | INTRODUCTION

An increasing number of clinical studies are showing that human mesenchymal stem cells (hMSCs) have great potential for treating various diseases such as impaired tissue functions and immunological disorders. hMSCs can be differentiated into several types of cells such as osteoblasts, adipocytes, chondrocytes, and neurons. Their secretome includes pro-angiogenic factors and growth factors, such as vascular endothelial growth factor, fibroblast growth factor 2, and insulin-like growth factor, which can promote tissue vascularization and regeneration.[1-3] They also secrete extracellular vesicles that encapsulate microRNAs and cytokines, which have been shown to modulate inflammation and promote the repair of damaged tissues.[4-6]

hMSCs are usually isolated from human tissues such as bone marrow, adipose tissue, or the umbilical cord and expanded in culture medium for clinical applications. However, in vitro aging of hMSCs happen and cells show limited proliferation, lose their differentiation capacity and immunosuppressive function after long-term expansion.[7,8] An ideal culture medium should maintain the characteristics of hMSCs, such as normal surface marker expression, and support their self-renewal after long-term culture. Although considered as nonessential supplements in a culture medium, nucleosides have been shown to influence the proliferation and differentiation of many mammalian cells including adipose-tissue-derived stem cells.[9-11] Whether nucleosides supplementation can affect the prolonged expansion and properties of hMSCs remains to be investigated.

Clinical applications of allogeneic hMSCs, which are derived from donors other than patients, require excessively large numbers of cells to treat hundreds or thousands of patients. In such cases, large-scale culture of hMSCs is necessary to obtain enough cells.[12] hMSCs for clinical studies are often cultured in multilayered culture flasks such as cell stack chambers. Although such planar culture platforms provide a much greater surface area for cell growth than single-layered tissue culture flasks, they have limited surface area, require intensive labor, and make process control, monitoring, and automation difficult.[13,14] Hollow fiber and fixed bed bioreactors can be used for high-density cell culture but have limited scalability for cell expansion and cause problems such as inhomogeneous nutrient supply and cell distribution.[12,15-18] Stirred tank bioreactor-based cell expansion, where cells are cultured under a mixed and controlled culture environment, exhibits good scalability and has the potential for culturing a large number of cells with controlled quality attributes.[14,19,20] In stirred tank bioreactor-based cell expansion, hMSCs are often inoculated on microcarriers, which are suspended in the bioreactor and provide the surface for cell adhesion and proliferation. Some studies reported the expansion of hMSCs with microcarriers in bioreactors, and the harvested cells from bioreactors maintained normal surface marker expression and the potential of differentiation into adipocytes, osteoblasts, and chondrocytes.[21-25]

An efficient method for the subculture of hMSCs in bioreactors is necessary for their large-scale manufacturing.[26] A common method involves removal of spent medium, microcarrier washing, proteolytic enzyme treatment for cell detachment from microcarriers, and separation of cells from microcarriers, followed by centrifugation, washing, and reinoculation of the harvested cells on fresh microcarriers. The whole process is laborious, susceptible to contamination, and potentially compromises cellular integrity by enzymatic digestion of cell membrane proteins and centrifugal forces.[27,28] Another method involves bead-to-bead cell transfer, which relies on the spontaneous migration of hMSCs from cell-populated microcarriers to fresh microcarriers that provide greater surface area for cell growth. Compared with the other methods, a bead-to-bead cell-transfer method can obviate the laborious process, risks of contamination, and cellular damage from proteolytic enzymes and physical stress during each subculture. However, after the supplementation of fresh microcarriers to cell-populated microcarriers, cell migration can be inefficient, resulting in a mixture of bare microcarriers and densely populated microcarriers or large microcarrier aggregates, which limited cell proliferation due to the shortage of the growth surface.[26,29,30] Large microcarrier aggregates can impede cell growth or cause cell death during expansion due to limited nutrient supply and encumber the enzymatic dissociation of hMSCs from microcarriers during cell harvest.[31,32]

We developed an efficient bead-to-bead cell-transfer method for the large-scale expansion of hMSCs with microcarriers in bioreactors for cell therapy. We first studied whether a medium with or without nucleosides can affect the expansion of hMSCs. Subsequently, we used different agitation profiles during bead-to-bead cell transfer to develop the subculture process in bioreactors. Finally, we used the medium containing nucleosides and bead-to-bead cell transfer for hMSC culture scale-up from 4- to 50-L in single-use stirred-tank bioreactors. The cell expansion and metabolite concentrations in bioreactors were monitored and the cells after the expansion were characterized.

## 2 | MATERIALS AND METHODS

### 2.1 | Expansion of hMSCs on planar surface

Bone-marrow derived hMSCs at passage 2 were purchased from Lonza, Inc. Alpha modification of minimum essential media with nucleosides (MEM alpha, nucleosides) (Gibco) was supplemented with 10% fetal bovine serum (FBS) (Biological Industries) and used as culture medium. hMSCs were plated on tissue culture flasks at a density of 3000

cells/cm$^2$ and culture in a 37°C incubator with 5% carbon dioxide for 6-7 days. Cells were dissociated from the culture vessels with TrypLE Select (Thermo Fisher Scientific). hMSCs at passage 3 were plated on 10-layer Nunc EasyFill Cell Factory (Thermo Fisher Scientific) vessels at the same plating density and cultured for 6-7 days, followed by enzyme treatment. The harvested cells (passage 4) were cryopreserved in liquid nitrogen for inoculation in spinner flasks or bioreactors.

To compare the effect of nucleosides in medium on the expansion of hMSCs, cells from two donors were cultured in tissue culture flasks (175 cm$^2$) from passage 3 to passage 6 in alpha modification of minimum essential media with (MEM alpha, nucleosides, Thermo Fisher Scientific) and without nucleosides (MEM alpha, no nucleosides, Thermo Fisher Scientific) supplemented with 10% FBS. hMSCs were also expanded in these two media in spinner flasks, as discussed in Section 2.2. The nucleosides included adenosine, cytidine, guanosine, uridine, 2′deoxyadenosine, 2′deoxycytidine HCl, 2′deoxyguanosine, and thymidine, all of which had a concentration of 10 mg/L in the medium. Cells were plated in tissue culture flasks at a density of 3000 cells/cm$^2$ and cultured for 7 days during each passage and harvested for cell count and analysis of surface marker expression.

## 2.2 | Expansion of hMSCs in spinner flasks

Forty milliliters of the culture medium was transferred into 125-mL disposable spinner flasks (Corning) and equilibrated in the incubator for 1 hour prior to cell inoculation. Suspension of Cytodex 1 (GE Healthcare) microcarriers in phosphate buffered saline without magnesium and calcium (PBS[−], Corning) were prepared according to the manufacturer's instruction and autoclaved. The microcarriers with a 500-cm$^2$ surface area were washed and suspended in 8 mL of fresh culture medium.

The cryopreserved hMSCs at passage 4 were thawed in a 37°C water bath then washed and suspended in fresh culture medium. The microcarriers suspension (500 cm$^2$) and a cell suspension with $1.5 \times 10^6$ hMSCs (2 mL) were transferred into the spinner flasks. The spinner flasks were placed in the incubator, agitated at 50 rpm for 6 cycles of 5-minute agitation and 25-minute static period. Subsequently, the spinner flasks were incubated without agitation overnight followed by adding 50 mL of culture medium and continuous agitation at 50 rpm from day 1 (Figure S1A). Duplicate 0.6-mL samples were taken daily from each spinner flask for cell counting and staining. The cells were counted using the NC-200 Automated Cell Counter, Via1-Cassette, and A100 and B buffers (ChemoMetec). Live/dead staining of the cells were done using a Cellstain R-Double Staining Kit (Dojindo) according to the manufacturer's instructions. After 6 days of culture, agitation was stopped to let the microcarriers settle, and 50% of the supernatant medium was replaced with fresh culture medium.

## 2.3 | Subculture of cells in spinner flasks

In a biosafety cabinet, the hMSCs cultured on microcarriers in the spinner flasks were swirled several times and 20 mL was transferred into new spinner flasks. Fresh microcarriers (400 cm$^2$) in the culture medium (80 mL) were added into the new spinner flasks. The new spinner flasks were placed in the incubator and agitated intermittently for 24 or 6 hours followed by continuously agitation or agitated continuously right after microcarrier addition, as shown in Table S1 and Figure S1B (supplemental information). Duplicate 0.6-mL samples of were taken daily from each spinner flask for cell counting and live/dead staining. Three images of cell staining per culture were used to calculate the ratios of cell-populated microcarriers in the cultures. Such bead-to-bead cell transfer were carried out on days 7 and 13, and each spinner flask culture was maintained for 26 days. Fifty percent of the medium was exchanged on day 19 and 23.

To compare the cell proliferation rate in suspension and planar cultures, passage 4 hMSCs were seeded in spinner flasks and tissue culture flasks (75 cm$^2$ T flasks, Corning) at the same seeding density (3000 cells/cm$^2$). The cells in the spinner flasks were subcultured using the intermittent 24 hours agitation mode, while the cells in tissue culture flasks were subcultured in a conventional manner that included enzymatic dissociation, centrifugation, and cell inoculation into new tissue culture flasks. Cells were cultured until they reached passage 9.

## 2.4 | Four liter cell culture in bioreactors

Two single-use 2-L bioreactors (UniVessel SU, Sartorius Stedim Biotech) were used for a 4-L culture of hMSCs. To equilibrate the culture medium, 1-L culture medium was put into each bioreactor and agitated under control at 37°C, pH 7.4, and 50% dissolved oxygen (DO) for 4 hours by using a BIOSTAT B (Sartorius Stedim Biotech). Afterwards, 2.27 g Cytodex 1 microcarriers and $3 \times 10^7$ hMSCs at passage 4 were inoculated into each bioreactor and intermittently agitated with 6 cycles of 5-minute agitation and a 25-minutes static period followed by overnight static phase without agitation. One liter of culture medium was added into each bioreactor and the working volume of each bioreactor increased to 2 L. From day 1 to day 8, the agitation rate was increased from 70 to 85 rpm to keep the microcarriers from settling. Fifty percent medium exchange was carried out on day 8.

## 2.5 | Scale-up to 20- and 50-L cultures

A Flexsafe STR 50-L culture bag (Sartorius Stedim Biotech) was set into a bag holder skid and 15 L of culture medium was transferred into the bag. The medium's temperature (37°C), pH (7.4), and DO (50%) were controlled using a BIOSTAT STR control tower for overnight. One-liter suspension of fresh microcarriers (18.2 g) in a culture medium and 4 L of culture on day 9 were transferred into the culture bag, and the culture volume became 20 L. Intermittent agitation was carried out for 24 hours (12 cycles of 5 minutes of agitation and 2 hours of static culture) to promote bead-to-bead cell transfer followed by continuous agitation of the culture for 4 days.

Subsequently, a 1-L suspension of fresh microcarriers (34.1 g) in the culture medium and 29 L of warmed fresh medium were added to the culture bag on day 13. The culture volume became 50 L, and intermittent agitation was carried out for 24 hours, similar to the 20-L culture. The 50-L culture was then agitated continuously for 14 days, during which 50% medium exchange was carried out on day 20. The agitation speed of the 50-L bioreactor was 50 rpm, which was converted into volumetric power inputs ($W/m^3$) according to a previous study.[33] Triplicate samples (3-5 mL) were taken from the bioreactor daily for cell count and live/dead double staining or stored in a −80°C freezer for metabolites analysis.

## 2.6 | Cell harvest

After the 50-L cell culture (day 27), 300-mL samples (approximately 0.34 g microcarriers) for cell harvest were taken from the bioreactor under agitation. Once the microcarriers settled, the supernatant medium was removed and PBS(−) was used to wash the microcarriers twice. Twenty milliliters of TrypLE Select enzyme solution (Thermo Fisher Scientific) was added to the microcarriers, which was put on a bioshaker (Taitec) and oscillated at 100 strokes per minute for 12 minutes at 37°C. Detachment of the cells from the microcarriers were confirmed under a phase contrast microscope (OLYMPUS). Culture medium (30 mL) was added to the suspension of detached cells and microcarriers, which was passed through a Falcon mesh (hole size: 50 μm; Corning) to separate the cells from microcarriers. The harvested cells were suspended at $5 \times 10^6$ cells/mL in STEM-CELLBANKER GMP grade (ZENOAQ RESOURCE Co, Ltd), aliquoted in 1.5-mL cryotubes (Thermo Fisher Scientific) and stored in BICELL freezing vessels (Nihon Freezer Co, Ltd) at −80°C for 24 hours. Subsequently, the frozen cells were transferred in a liquid nitrogen tank and used for characterization.

## 2.7 | Characterization of expanded cells

To confirm their growth potential, expanded cells were thawed, plated onto tissue culture flasks, and cultured for 8 days. Their surface antigens were analyzed using a BD Stemflow hMSC Analysis Kit and BD FACSVerse flow cytometer (BD Biosciences). Cells incubated with hMSCs' positive antibody cocktail (CD90 FITC, CD105 PerCP-Cy5.5, and CD73 APC) and negative antibody cocktail (CD34 PE, CD11b PE, CD19 PE, CD45 PE, and HLA-DR PE) were used as the experiment group. Cells treated with both positive isotype control cocktail (mIgG1 FITC, mIgG1 PerCP-Cy5.5, and mIgG1 APC) and negative isotype control cocktail (mIgG1 PE and mIgG2a PE) were used as the isotype controls. Harvested cells were also stained with CD44 PE from the same kit and CD146 APC (BioLegend) for flow cytometry.

To confirm their differentiation potential, harvested cells were cultured in adipogenic medium or osteogenic medium (PromoCell) for 2 weeks, or chondrogenic differentiation medium (PromoCell) for 3 weeks according to the manufacturer's instructions. After being cultured in the differentiation medium, samples were washed with PBS (−) and fixed with 4% paraformaldehyde (Wako). Samples incubated in adipogenic, osteogenic, and chondrogenic differentiation media were stained with Oil Red O solution, Alizarin Red S solution, and Alcian Blue solution, respectively. Stained samples were washed three times before imaging.

Mixed lymphocyte reaction assay was used to study the immunomodulatory properties of expanded hMSCs. In each well of 24-well plates (Corning), $1 \times 10^5$ peripheral blood mononuclear cells (PBMCs, HemaCare) were cultured with 0, $1 \times 10^3$, $5 \times 10^3$, $1.25 \times 10^4$, $2.5 \times 10^4$ or $5.0 \times 10^4$ hMSCs (sample name: PBMCs only, 100 to 1, 20 to 1, 8 to 1, 4 to 1 or 2 to 1) for 5 days. PBMCs were stimulated with Dynabeads Human T-Expander (Thermo Fisher Scientific) and 200 IU/mL recombinant IL-2 (Proleukin, Norvartis) in RPMI medium 1640 (Thermo Fisher Scientific) with 10% heat inactivated FBS. After the coculture, PBMCs were collected, counted, and stained with CD3 PE antibody (Beckman Coulter) for flow cytometry.

## 2.8 | Metabolite analysis

Frozen medium samples from bioreactors were thawed to room temperature and centrifuged at 500*g* for 5 minutes, and the supernatant was used for metabolite analysis. All the medium samples were analyzed using the BioProfile FLEX Automated Cell Culture Analyzer (Nova Biomedical) to quantify the concentrations of glutamine, glutamate, glucose, and lactate. Medium samples taken from the 20- and 50-L cultures (day 9-27) were also analyzed using the C2MAP Cell Culture Media Analysis Platform (Shimadzu) to quantify 95 components in the medium including sugars, nucleic acid-associated compounds, amino acids, vitamins, and other metabolic intermediates. The C2MAP system is composed of an auto-sampler and ultrafast liquid chromatograph mass spectrometer (LC/MS/MS). The auto-sampler pretreats medium samples with deprotein reagents and internal standard, filters precipitated proteins, and dilute samples. The LC/MS/MS module separates individual components in pretreated samples and detects 95 separated components with high sensitivity. C2MAP Trends software was used to obtain the concentration of each component. The specific rates of nucleoside consumption in units of mg of nucleoside/million cells/day were calculated according to a previous work.[24]

## 2.9 | Statistical analysis

Cell expansion experiments in monolayer culture and spinner flasks were run in triplicate. Results were presented as mean of three samples or mean ± SD. One-way or two-way analysis of variance (ANOVA) followed with Tukey post hoc tests was used to analyze the difference between the means of more than two groups. *P* values less than .05 were considered statistically significant.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Effect of nucleosides on expansion of hMSCs

Although many specialized media for hMSCs are now commercially available, we used the widely available MEM alpha with 10% FBS for cell expansion. We first investigated the effect of nucleosides in MEM alpha on cell proliferation and surface marker expression of hMSCs. The culture media with and without nucleosides were used for the planar culture of hMSCs from passage 4 to passage 6 in tissue culture flasks. The cells were harvested and counted after culturing for 7 days per passage. Compared with the medium without nucleosides, the medium containing nucleosides resulted in a significantly higher number of cells of one donor from passage 4 to passage 6 and significantly higher number of cells of another donor from passage 4 to passage 5 (Figure 1A). Flow cytometry analysis of harvested cells showed that the cells cultured without nucleosides from passage 4 to passage 6 had lower expression of CD73 (83.8% vs 99.6%), CD90 (85.8% vs 99.4%), and CD105 (85.2% vs 99.1%) and higher expression of hMSCs' negative surface markers (4.80% vs 0.06%) compared with those cultured with nucleosides (Figure 1B). The suspension culture of hMSCs with Cytodex 1 microcarriers in spinner flasks also showed a higher cell proliferation rate in the medium with nucleosides than those in the medium without nucleosides (Figure 1C). These results suggest that supplementation of nucleosides in medium can promote proliferation and maintain the immunophenotype of hMSCs during their prolonged culture.

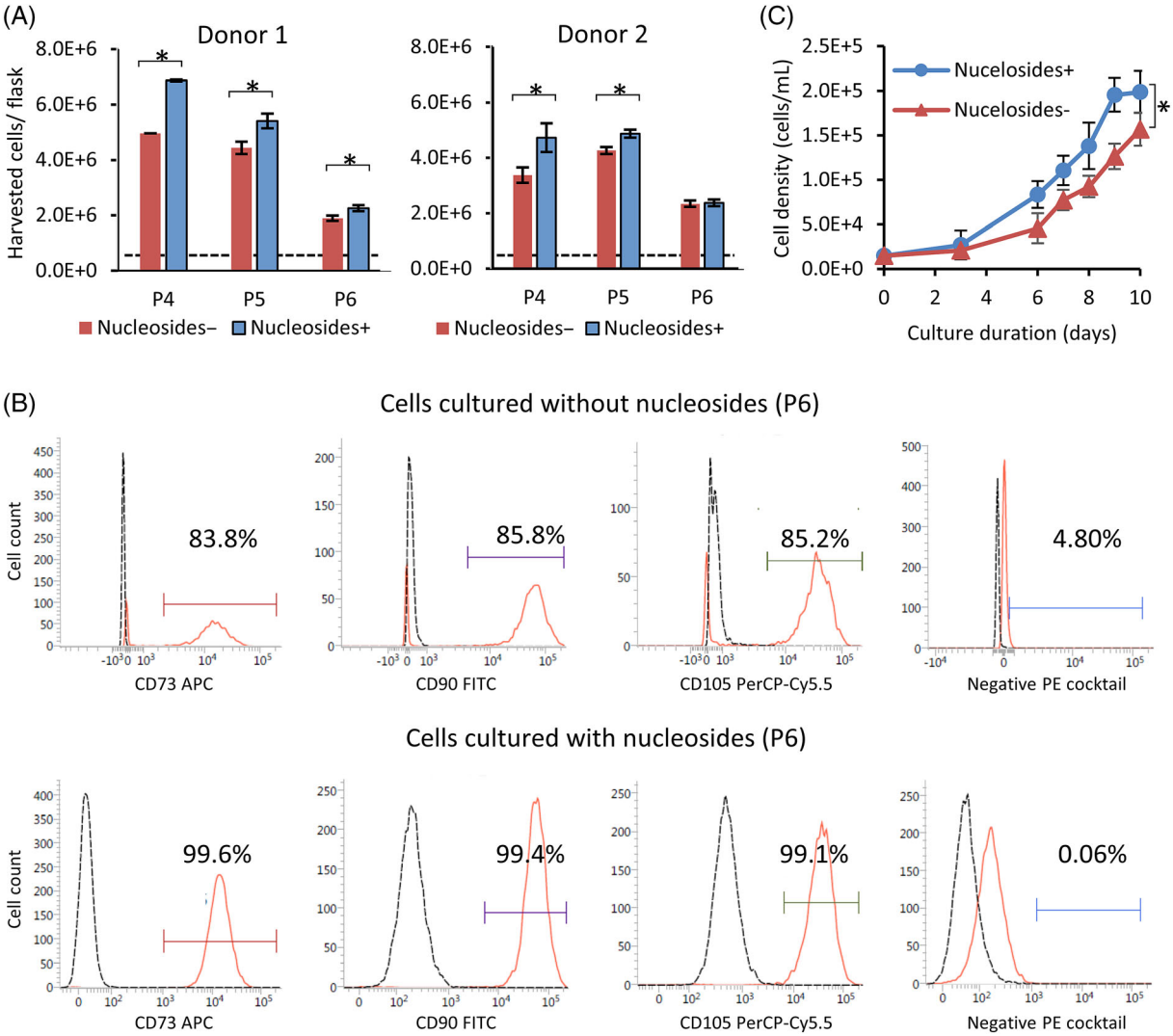


**FIGURE 1** Effect of nucleosides in culture medium on proliferation and surface marker expression of hMSCs. A, Number of cells harvested from tissue culture flasks (175 $cm^2$). Cells from two donors were cultured from passage 4 to passage 6 in αMEM/10% FBS with and without nucleosides. Dash lines represent number of cells seeded ($5.25 \times 10^5$) per flask. B, Expression of surface markers on passage 6 cells cultured without and with nucleosides. Red solid lines represent cells treated with negative and positive antibody cocktails and dashed lines represent cells treated with negative and positive isotype controls. C, Proliferation of cells with Cytodex 1 microcarriers in 100-mL spinner flasks. Experiments were run in triplicate; mean ± SD. Asterisk * indicates significant statistical difference ($P < .01$, Tukey post hoc tests after two-way ANOVA)

### 3.2 | Effect of agitation mode on bead-to-bead cell transfer

Next, we used different agitation profiles during bead-to-bead cell transfer to develop the expansion process in bioreactors. We hypothesized that if cell-populated microcarriers are in contact with fresh microcarriers for a sufficient amount of time, cell migration between them can be promoted. Therefore, we checked whether intermittent agitation or continuous agitation affects cell transfer, as described in Materials and Methods and Table S1. Fresh microcarriers were added to cell-populated microcarriers that had been cultured in spinner flasks, as shown in Figure 2A. Subsequently, the spinner flasks were

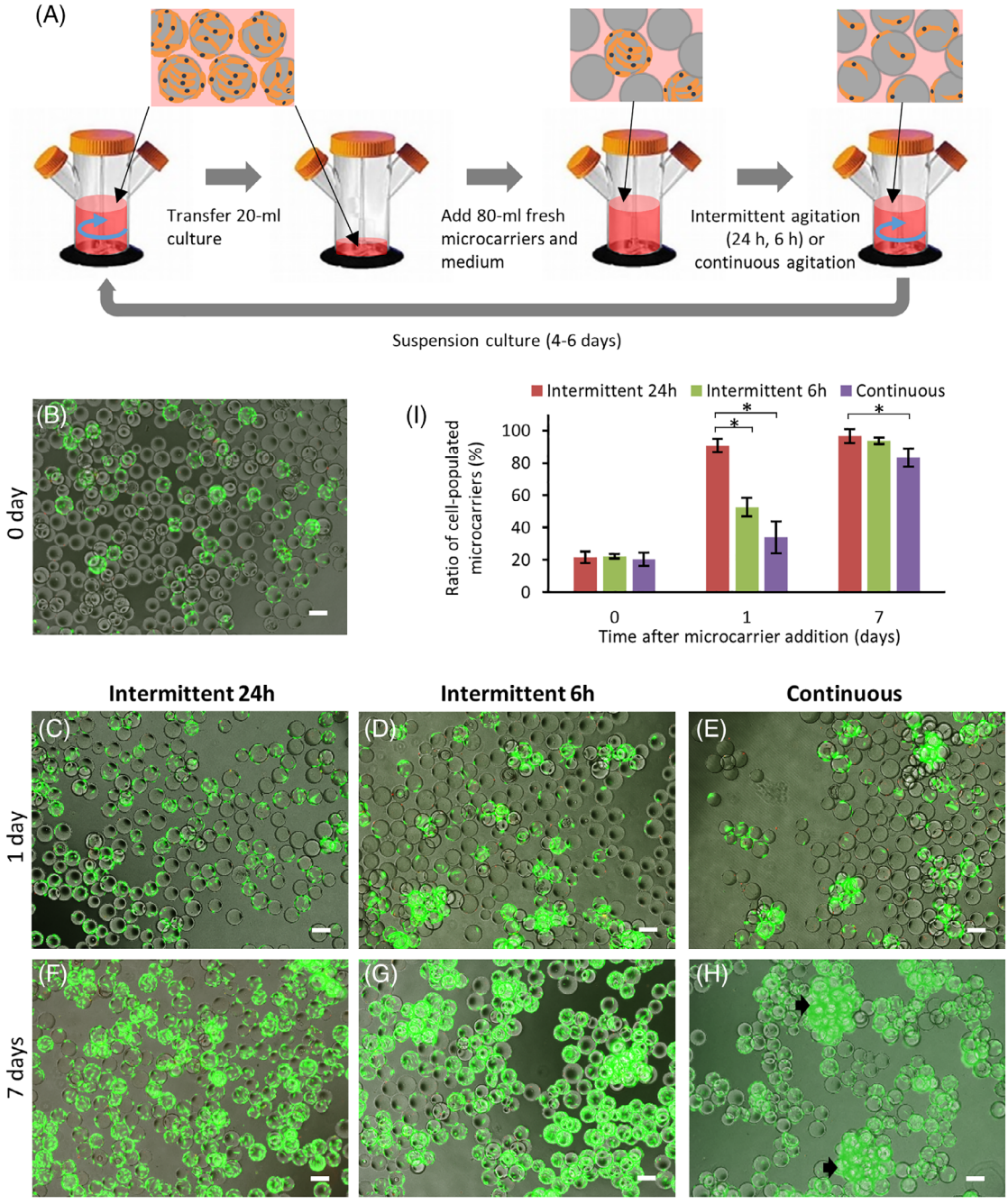


**FIGURE 2** Effect of agitation mode on bead-to-bead cell transfer after microcarrier addition to spinner flasks. A, Different agitation modes used during bead-to-bead cell transfer. B–H, Staining of cells (live cells: green; dead: red) on beads after microcarrier addition. B, Samples taken immediately after microcarrier addition (day 0). C–E, Samples taken on day 1 after microcarrier addition. F–H, Samples taken on day 7 after microcarrier addition. C and F are from intermittent 24 hours group, D and G are from intermittent 6 hours group, and E and H are from continuous group. Arrows in H show large microcarrier aggregates. Scale bar = 200 μm. I, Ratio of cell-populated microcarriers in cultures that underwent different agitation modes. Samples were taken on days 0, 1, and 7 after microcarrier addition. Experiments were run in triplicate; mean ± SD; *$P$ < .01, Tukey post hoc tests after one-way ANOVA

agitated intermittently for 24 or 6 hours followed by continuous agitation or agitated continuously right after microcarrier addition. Cell-staining images of 1 day post microcarrier addition showed that 24-hour intermittent agitation resulted in 90.7 ± 4.2% of microcarriers populated by cells, while 6-hour intermittent agitation resulted in 52.7 ± 5.7% of microcarriers populated by cells (Figure 2C,D,I). The continuous agitation mode had a much lower ratio of microcarriers (34.0 ± 9.8%) populated by cells (Figure 2E,I). These results suggest that intermittent stirring after microcarrier addition can promote cell migration to fresh microcarriers. During the intermittent agitation, cell-populated microcarriers (old microcarriers) and fresh microcarriers settle so that hMSCs were in contact with both microcarriers. Therefore, some of migrating cells transferred from old microcarriers to nearby fresh microcarriers. Multiple cycles of intermittent agitations could allow a homogeneous mixing of these microcarriers, thus homogeneous cell distribution after cell migration. After 7 days of culturing, cells adhered and proliferated on 96.7 ± 4.2% of the microcarriers in the intermittent 24 hours group, 93.7 ± 2.1% of the microcarriers in the intermittent 6 hours group, and 81.3 ± 5.5% of the microcarriers in the continuous group (Figure 2F-I).

In addition, the continuous group had microcarrier aggregates that were densely populated by cells and microcarriers with few cells (Figure 2H). Intermittent agitation after microcarrier addition resulted in much less microcarrier aggregates in comparison (Figure 2F,G). Due to inefficient cell migration in the continuous group, microcarriers with confluent cells and bare microcarriers co-existed in suspension culture, and the former were more likely to precipitate and aggregate with each other, forming large microcarrier aggregates. In contrast, the intermittent agitation facilitated cell migration and homogeneous cell distribution on microcarriers, which had similar density and could be homogeneously dispersed in medium to avoid the formation of large aggregates. Large aggregates of cell-microcarriers are unfavorable because they can limit the transfer of nutrients and the removal of waste product from cells within them, which adversely affect the growth and quality of cells.[13]

During the 26-day cultures in spinner flasks, fresh microcarriers and medium were added twice to cell-populated microcarriers for bead-to-bead cell transfer, as shown in Figure 2A. The proliferation of cells in the spinner flasks is shown in Figure 3A. When the cell densities were low, cells cultured under different agitation modes had similar proliferation rates. As the cell density increased, cell growth in the continuous group had a propensity to grow slower than the other two groups, as shown on day 13 and 26. The inferior cell growth in the continuous group may be explained by the limited surface area the cells could use since much less microcarriers were occupied by cells in this group. In addition, large microcarrier aggregates in the continuous group might have limited the mass transfer for the cells within the aggregates, which could inhibit cell growth.[13]

After expansion in spinner flasks for 26 days, the cells were treated with an enzyme to dissociate them from the microcarrier surface. While cells could be dissociated efficiently from microcarriers in the intermittent 24 hours and intermittent 6 hours groups, large cellular clumps were trapped between microcarriers in the continuous group (Figure 3B-D). By comparing the amount of cells harvested and that of cells estimated from the sampling before harvest, the harvest efficiency was calculated. The harvest efficiency was significantly lower in the continuous group (59.8 ± 11.1%) than intermittent 24 hours (92.0 ± 1.4%) and intermittent 6 hours (91.4 ± 4.0%) groups (Figure 3E). The formation of large microcarrier aggregates in the continuous group limited enzyme penetration into microcarrier aggregates, which could hamper cell dissociation from microcarriers.

Serial cell subculture in spinner flasks and tissue culture flasks were carried out to compare the cell growth in the two culture platforms (Figure 3F,G). The hMSCs of passage 4 were seeded in both spinner flasks and tissue culture flasks and subcultured until they reached passage 9. Bead-to-bead cell transfer was used for serial suspension culture while enzyme-based cell dissociation was used for serial subculture in the tissue culture flasks. The results showed that the conventional subculture in the tissue culture flasks had lower cell proliferation rates than the subculture based on bead-to-bead cell transfer in the spinner flasks (Figure 3F). The doubling time of cells in the tissue culture flasks increased from 47.8 hours at passage 4 to 76.3 hours at passage 9, while the doubling time of cells in the spinner flasks only had a modest increase (43.6 hours at passage 4 and 50.3 hours at passage 9) (Figure 3G). From passage 7 to passage 9, the doubling times of cells in spinner flasks were significantly shorter than that in tissue culture flasks. The enzyme treatment, centrifugation, and reseeding in the conventional subculture could cause cleavage of cell membrane proteins and physical stress-associated cell damage, which could adversely affect cell growth. Proteolytic enzyme treatment has been found to alter cell behaviors such as the expression of proteins related to cell metabolism, growth regulation, and differentiation.[34-37] Under the intermittent agitation modes, migrating cells transferred from old microcarriers to adjacent fresh microcarriers without enzyme detachment, which had a faster cell-proliferation rate compared with conventional enzyme-based planar culture.

### 3.3 | Cell expansion and subculture in bioreactors

As intermittent agitation after microcarrier addition could promote bead-to-bead cell transfer, it was adopted for the expansion of cell culture in bioreactors, as shown in Figure 4A. The agitation rates of bioreactors were converted into volumetric power inputs and shown in supplemental Table S2. After hMSCs were expanded with microcarriers in the 4-L culture for 9 days, the cell-populated microcarriers along with the medium in the culture were transferred into a stirred tank bioreactor with a maximum working volume of 50 L, followed by adding fresh microcarriers and medium and intermittent agitation of the resultant 20 L of culture for 24 hours. The 20-L culture was then agitated continuously for 3 days, and fresh microcarriers and medium were added again to the bioreactor to initiate a 50-L culture. The 50-L culture was agitated intermittently for 24 hours followed by continuous agitation until day 27. Figure 4B showed that after expansion in the 4-L culture for 9 days, the cells became nearly confluent on the microcarrier surface. After addition of fresh microcarriers and intermittent agitation,

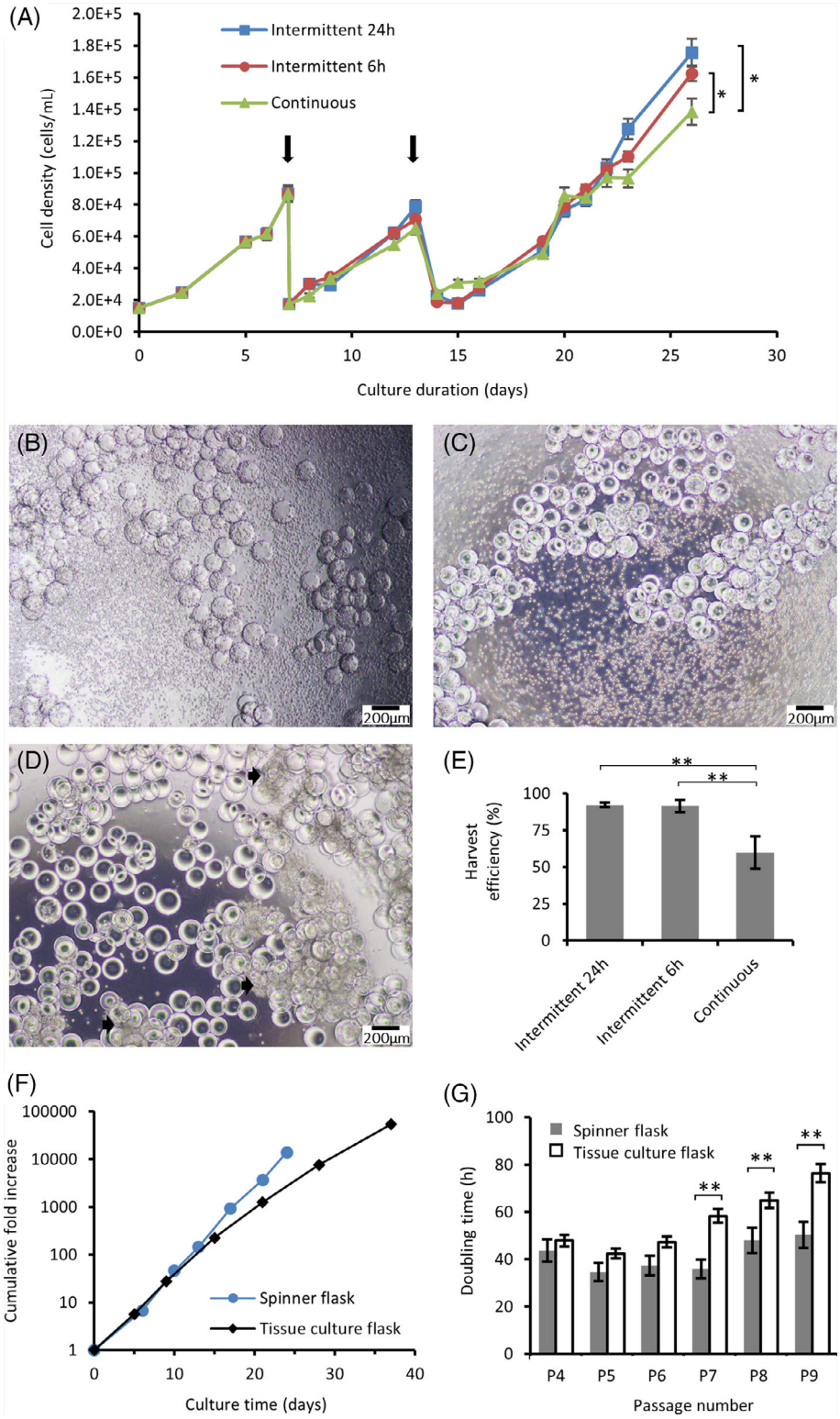


**FIGURE 3** Proliferation and harvest of hMSCs cultured with different agitation modes during passaging and comparison of suspension culture with planar culture. A, hMSC proliferation in cultures underwent three agitation modes. Cells were cultured with microcarriers for 26 days during which fresh microcarrier suspensions were added on days 7 and 13 (marked with arrows). Data points represent average cell densities of triplicate spinner flask cultures (N = 3). B-D, Phase contrast images taken after enzyme treatment of cells subcultured with intermittent 24 hours, intermittent 6 hours, and continuous agitation modes, respectively. Arrows in D mark cell clumps trapped in aggregated microcarriers. E, Harvest efficiency of cells from microcarriers. F–G, Cumulative fold increase and cell doubling times of serial subcultures in spinner flasks and tissue culture flasks. Experiments were run in triplicate; $^{*}P < .01$; $^{**}P < .001$; Tukey post hoc tests after one-way ANOVA (A and E) or two-way ANOVA (G)

fluorescent microscopy showed that most of the microcarriers became populated with cells and few cell-microcarrier aggregates formed (day 10 and 14). After their migration onto fresh microcarriers, cells proliferated on the microcarrier surface under continuous agitation until confluence (day 13 and 27). High viability of cells during the expansion was maintained as few dead cells (red) were found.

The cell density increased over time in the 4-, 20-, and 50-L cultures and reached a final cell density of $2.9 \times 10^5$ cells/mL on day 27 (Figure 5A). Although cell viability dropped transiently after cell inoculation and microcarrier addition, cell viability was maintained above 83.7% during the entire culture period, and the final cell viability reached 98.1%. The transient drop of cell viability could have

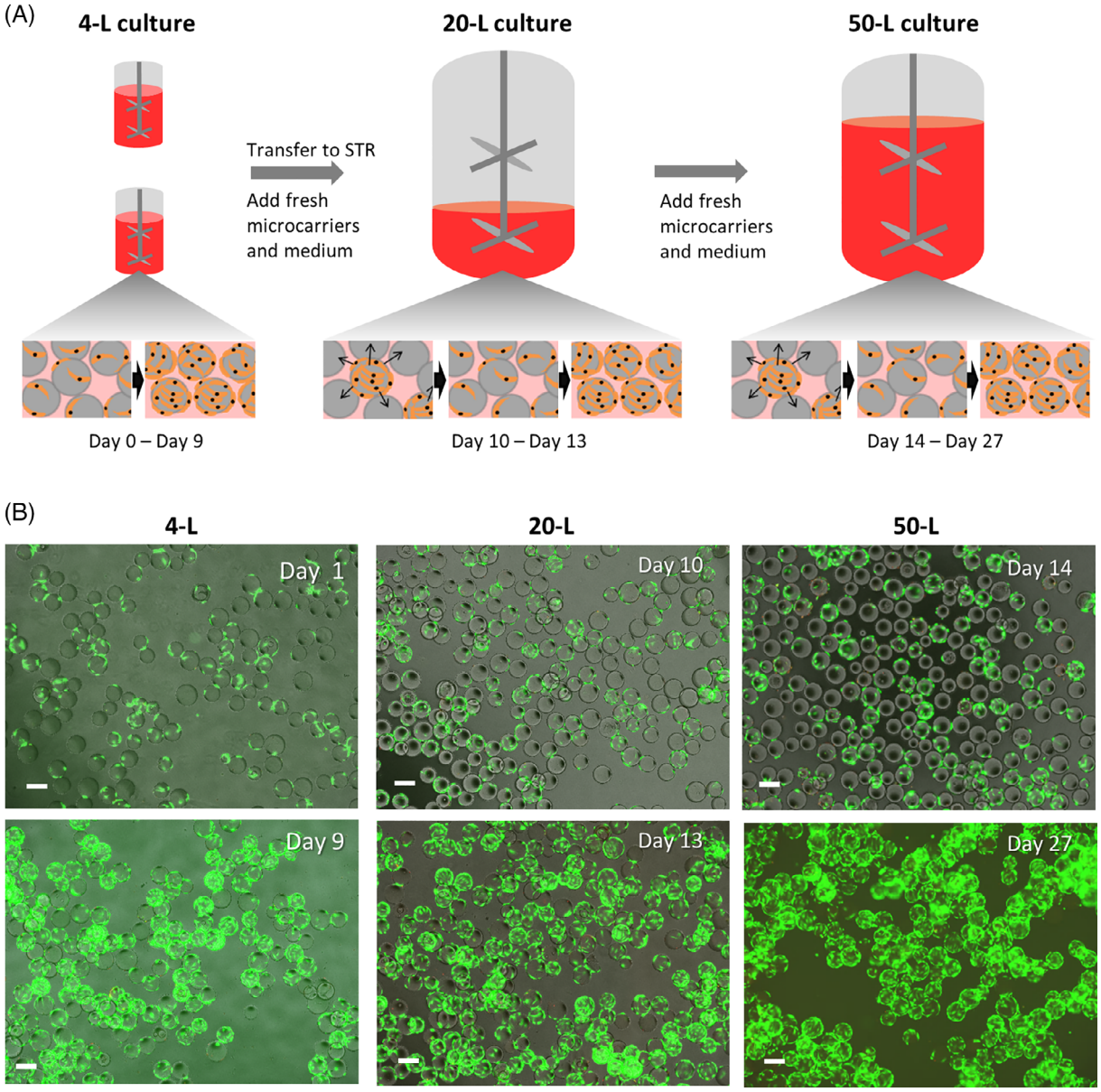


**FIGURE 4** Serial subculture from 4- to 50-L cultures in bioreactors. A, Subculture of hMSCs in stirred-tank bioreactors (STR). B, Fluorescent microscopy of cells (live cells: green; dead cells: red) on microcarriers sampled from 4-, 20-, and 50-L cultures in bioreactors. Left: samples taken on days 1 and 9 from 4-L culture; middle: samples taken on days 10 and 13 from 20-L culture; right: samples taken on days 14 and 27 from 50-L culture. Scale bar = 200 μm. Fresh microcarriers were added on days 9 and 13 followed by 24-hour intermittent agitation to facilitate bead-to-bead cell transfer

resulted from sheer stress-associated cell damage after cell inoculation or limitation of mass transfer to cells when microcarriers settled during the intermittent agitation. Such issues may be solved using a lower agitation rate or shorter static period during the intermittent agitation. The total number of cells increased from $6.00 \times 10^7$ to $1.45 \times 10^{10}$ (Figure 5B), with a 242-fold increase in total number of cells during the 27-day culture.

From these results, we confirmed that microcarrier addition followed by intermittent agitation can promote cell migration to new microcarriers and support serial subculture without enzymes and large-scale expansion of hMSCs in bioreactors. Most studies on the microcarrier-based culture of hMSCs in stirred-tank bioreactors reported inoculation and harvest of cells in one batch within 14 days, during which cells had a 12 to 36-fold increase.[38-42] Attempts to use bead-to-bead cell transfer for cell passaging and expansion often resulted in low efficiency of cell transfer, and many microcarriers either fully confluent or completely without cells.[26,29] Takahashi et al. reported that using bead-to-bead cell transfer for hMSCs led to a 20-fold cell increase during their 45-day culture in 10-mL spinner flasks.[30] Lawson et al. reported two feeds of fresh microcarriers and medium to an ongoing 20-L culture to increase the scale to 50 L and achieved a 43-fold cell increase in 11 days.[40] Their 50-L culture

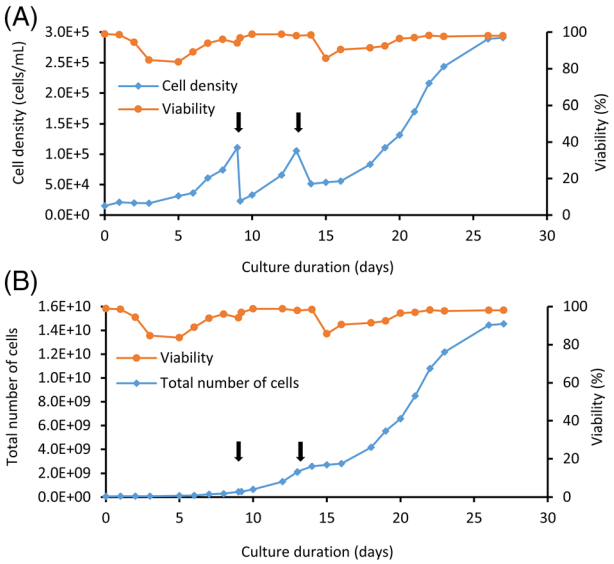


**FIGURE 5** Cell density (A) and calculated total cell number (B) during serial culture in bioreactors. Days 0-9:4-L culture; days 9-13:20-L culture; days 13-27:50-L culture. Arrows mark addition of fresh microcarrier suspension. Data points represent average cell density or cell number calculated from three samples

showed faster cell proliferation than in our study, possibly because cells at lower passage (passage 2), collagen-coated microcarriers, and human platelet lysate were used for cell expansion. In our study, we carried out serial subculture of passage 4 hMSCs from 4- to 50-L in bioreactors with bead-to-bead cell transfer and obtained much greater increase during cell expansion. Since our serial subculture in spinner flasks achieved more than a 10 000-fold increase in cell number, we believe that we can further increase the scale of bioreactor culture with our bead-to-bead cell-transfer method. We also used higher microcarrier densities such as 6.8 g of Cytodex 1 per liter for cell expansion and found that the migration of cells to microcarriers occurred at higher microcarrier densities. These results suggest that our bead-to-bead cell-transfer method can be used for high-density cell expansion in stirred bioreactors.

## 3.4 | Metabolite concentration during cell expansion

The concentrations of glutamine, glutamate, glucose, and lactate of the culture medium in bioreactors were analyzed using the BioProfile FLEX Automated Cell Culture Analyzer (Figure 6). During the first 20 days of cell expansion, glucose and glutamine concentrations were higher than one third of those in fresh culture medium. With an increase in cell density from day 20 to day 27, nutrient consumption by cells accelerated, with glucose concentration dropping to 0.36 mM and glutamine concentration dropping to 0.35 mM. Glutamate concentration remained relatively stable throughout the culture period, indicating its consumption by the cells was negligible. The final concentration of lactate increased to 12.77 mM but did not compromise cell viability. These results are in agreement with previous studies that showed the growth of hMSCs was not inhibited until lactate concentration was above 35.4 mM.[43]

Medium samples taken from 20- and 50-L cultures (day 9-27) were also analyzed using the Cell Culture Media Analysis Platform to quantify 95 components in the medium including sugars, nucleic acid-associated compounds, amino acids, vitamins, and other metabolic intermediates. The changes in glucose, glutamine, and lactate concentrations detected with this platform correlated very well with those measured from the former analyzer (Figure 6B). Interestingly, we found that the decrease in the concentrations of cytidine, thymidine, deoxycytidine, and adenosine showed a similar trend with those of glucose and glutamine, and depletions of cytidine and deoxycytidine within 4 days after adding fresh medium or medium exchange, suggested the consumption of nucleosides by cells (Figure 6B and Figure S2). The specific rates of nucleoside consumptions for these four nucleosides in the 50-L bioreactor were similar, as shown in Table S3. Although other four nucleosides were contained in the culture medium, 2′deoxyadenosine and 2′deoxyguanosine were not quantified because parameters for their detection were not provided by the analyzer manufacturer. Uridine and guanosine were not detected in all samples from the whole culture period, possibly because their concentrations in medium was below the detection limit. However, it is possible that these undetected nucleosides were also used by cells during their expansion in bioreactors. The consumption of the nucleosides by hMSCs, along with the previous data that showed superior cell proliferation rates in medium with nucleosides, further suggests that nucleosides should be beneficial for the expansion of hMSCs.

## 3.5 | Cell harvest and characterization

We harvested hMSCs after their expansion in 50-L culture in bioreactors to check if they maintained their characteristics. Figure 7A shows images of microcarrier samples taken before and after cell harvest. Before harvest, cells reached confluence on most of the microcarriers. After enzyme treatment and separation of cells from the microcarriers, few cells could be observed on the surface of the microcarriers. When the harvested cells were plated and cultured on tissue culture flasks, they attached and proliferated during the 8-day culture (Figure 7B). Flow cytometry analysis showed that more than 98.1% of the expanded cells had the expression of CD90, CD73 and CD105, and less than 0.2% had the expression of CD34, CD11b, CD19, CD45 or HLA-DR (Figure 7C). After cell expansion, 95.7% and 94.0% of the cells expressed CD44 and CD146, respectively (Figure S3). The hyaluronan receptor CD44 can facilitate the migration of MSCs to wound sites for tissue regeneration.[44] Expression of CD146 on hMSCs correlated with higher multipotency, immunomodulatory and secretory potency.[45,46]

After 2 weeks of adipogenic and osteogenic differentiation of the harvested cells, Oil Red O staining showed the formation of oil droplets and Alizarin Red S staining showed the extracellular matrix

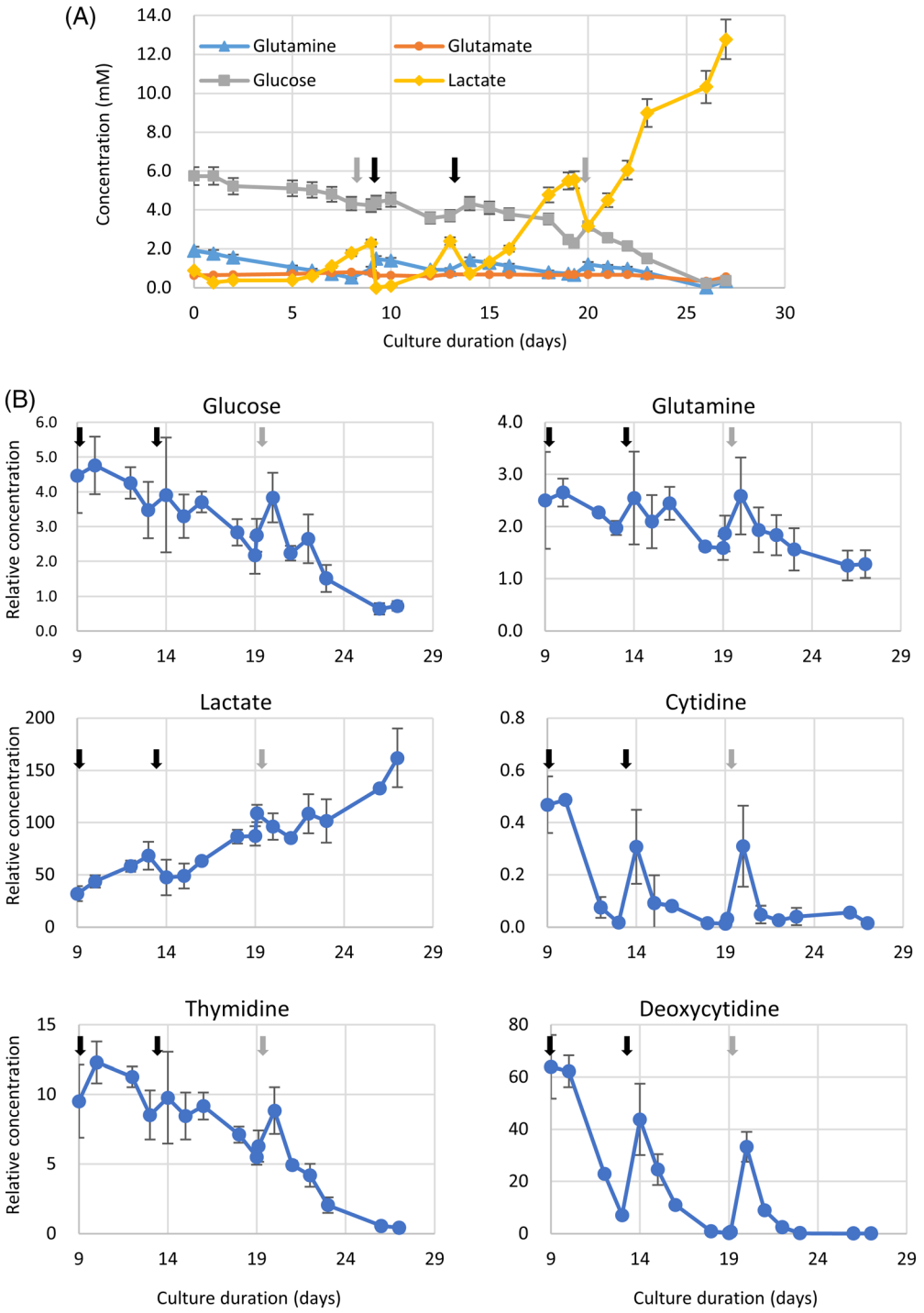


**FIGURE 6** Concentration of metabolites measured using two medium analyzers. A, BioProfile FLEX Automated Cell Culture Analyzer, B, Cell Culture Media Analysis Platform. Days 0-9:4-L culture; days 9-13:20-L culture; days 13-27:50-L culture. Black arrows mark addition of fresh microcarrier suspension (microcarrier concentration = 5000 $cm^2$/L). Gray arrows mark 50% medium exchange. Data points represent average metabolite concentrations measured from three medium samples

mineralization (Figure 7D). After 3 weeks of chondrogenic differentiation of pellets from the harvested cells, cartilaginous tissue formed, as shown by Alcian Blue staining of sulfated proteoglycans. The hMSCs expanded in bioreactors were cultured with PBMCs for 5 days and T cell proliferation was evaluated (Figure 7E and Figure S4). T cell proliferation was suppressed in the presence of hMSCs and T cell growth was more inhibited with higher number of hMSCs. These results suggest that the expanded cells maintained their proliferation ability, differentiation potential and immunomodulatory property of hMSCs.

In this study, cells expanded without nucleosides in planar culture had decreased proliferation rate and lower level of hMSC's surface markers, which could be the result of chromosomal aberrations in in vitro aging. Previous studies on the expansion of hMSCs also showed lower expression levels of their surface makers accompanied with genomic instability of aging hMSCs.[8] Chromosomal aberrations including translocations and deletions were reported in bone marrow-derived hMSCs after their repeated division.[47] On the other hand, studies showed that nucleotide deficiency in cells could promote genomic instability

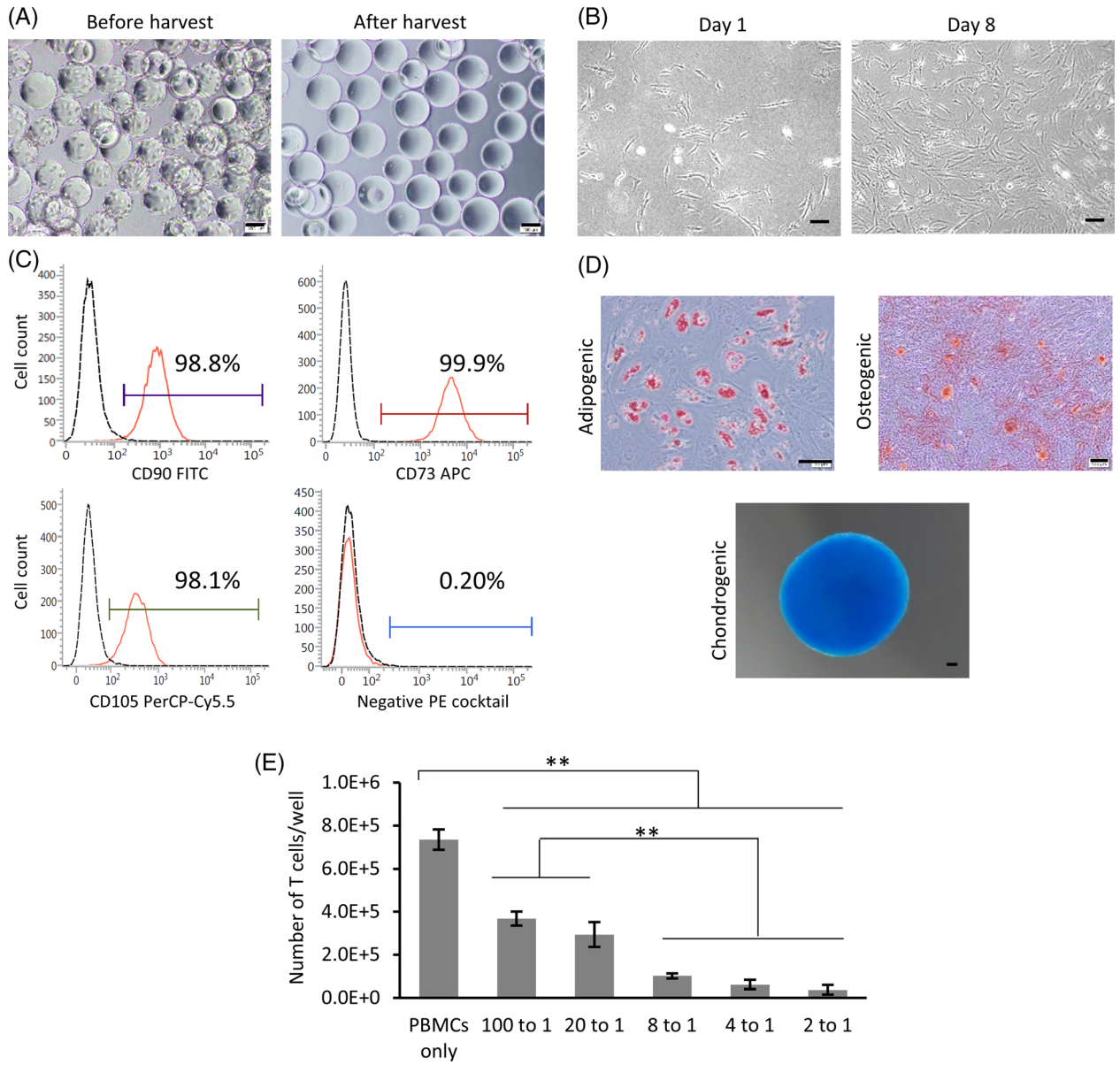


**FIGURE 7** Harvest of cells from microcarriers and characterization of harvested cells. A, Microscopic images of microcarriers before and after cell harvest. B, Proliferation of harvested cells in tissue culture flask. C, Flow cytometry analysis of surface markers of harvested cells. Red solid lines represent cells treated with negative and positive antibody cocktails and dashed lines represent cells treated with negative and positive isotype controls. D, Staining of adipogenic, osteogenic, and chondrogenic differentiation of harvested cells. Scale bar = 100 μm. E, The number of T cells per well after culturing PBMCs with different ratio of hMSCs (PBMCs:hMSCs = 100 to 1, 20 to 1, 8 to 1, 4 to 1, and 2 to 1). PBMCs cultured without hMSCs were used as a control group. Experiments were run in triplicate; Mean ± SD. $^{**}P < .001$, Tukey post hoc tests after one-way ANOVA

during DNA replication while exogenously supplied nucleosides increased the nucleotide pool and rescued DNA damage.[48] A recent study showed that nucleosides supplementation in reprogramming medium can reduce the DNA damage and genomic rearrangements on induced pluripotent stem cells.[49] Therefore, it is possible that the nucleosides in our culture medium reduced the genetic aberrations in hMSCs during their expansion. Future work will be done to confirm the genomic stability of hMSCs after their expansion with exogenous nucleosides and the enzymes that involve with nucleoside metabolism.

## 4 | CONCLUSION

We investigated the effect of nucleosides in medium on the proliferation of hMSCs and the effect of different agitation modes on bead-to-bead cell transfer in suspension culture. We also developed the serial subculture process toward 50-L suspension culture of hMSCs in bioreactors. Planar and suspension cultures showed that the culture medium with nucleosides promoted cell proliferation and maintained the immunophenotype of hMSCs compared with those without nucleosides. Our bead-to-bead cell transfer method, which involved adding

fresh microcarriers to the suspension of cell-populated microcarriers followed by intermittent agitation, facilitated spontaneous cell migration to fresh microcarriers, improved cell proliferation, and inhibited microcarrier aggregations compared with continuous agitation. Such an enzyme-free subculture enabled faster proliferation of hMSCs in suspension culture than those in planar culture. With this method, we subcultured the hMSCs in bioreactors from 4 to 50 L, achieved 242-fold increase in cell number to $1.45 \times 10^{10}$ cells after 27-day culture. Cells cultured in the bioreactors consumed nucleosides and maintained their proliferation ability, expression of their surface markers, tri-lineage differentiation potential and immunomodulatory property. These results indicate that nucleosides can promote hMSC expansion and that our bead-to-bead transfer method for microcarrier-based cell expansion in bioreactors has the potential for large-scale manufacturing of cell and gene therapy products. To move our method toward clinical application, future work, such as on xeno-free cell expansion, thorough removal of microcarriers from cells, and analysis of therapeutic potency-related cell attributes, wash, concentration, and cryopreservation of a large number of cells, should be conducted to ensure the safety and efficacy of final cellular products.

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## CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

## AUTHOR CONTRIBUTIONS

S.C.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; Y. Sato, Y. Suzuki: collection and/or assembly of data; Y.T., R.T., M.O., K.N.: data analysis and interpretation, administrative support.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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