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Human amniotic mesenchymal stromal cells support the ex vivo expansion of cord blood hematopoietic stem cells

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Abstract

Currently over 30 000 allogeneic hematopoietic stem cell (HSC) transplantations have been performed for the treatment of hematological and nonhematological diseases using HSC from umbilical cord blood (CB). However, the wide utilization of CB as a source of HSC is limited by the low number of cells recovered. One strategy to expand ex vivo CB-HSC is represented by the use of bone marrow mesenchymal stromal cells (BM-MSCs) as a feeder to enhance HSC proliferation while maintaining HSC stemness. Indeed, BM-MSCs have been recognized as one of the most relevant players in the HSC niche. Thus, it has been hypothesized that they can support the ex vivo expansion of HSC by mimicking the physiological microenvironment present in the hematopoietic niche. Due to the role of placenta in supporting fetal hematopoiesis, MSC derived from the amniotic membrane (hAMSC) of human term placenta could represent an interesting alternative to BM-MSC as a feeder layer to enhance the proliferation and maintain HSC stemness. Therefore, in this study we investigated if hAMSC could support the ex vivo expansion of HSC and progenitor cells. The capacity of hAMSCs to support the ex vivo expansion of CB-HSC was evaluated in comparison to the control condition represented by the CB-CD34$^+$ cells without a feeder layer. The coculture was performed at two different CD34$^+$:MSC ratios (1:2 and 1:8) in both cell-to-cell contact and transwell setting. After 7 days, the cells were collected and analyzed for phenotype and functionality. Our results suggest that hAMSCs represent a valuable alternative to BM-MSC to support: (a) the ex vivo expansion of CB-HSC in both contact and transwell systems, (b) the colony forming unit ability, and (c) long-term culture initiating cells ability. Overall, these findings may contribute to address the unmet need of high HSC content in CB units available for transplantation.

KEYWORDS
bone marrow mesenchymal stromal cells, cord blood, ex vivo expansion, hematopoietic stem cells, human amniotic mesenchymal stromal cells

V. Orticelli and A. Papait contributed equally to this study.

Significance statement

To date, more than 30 000 allogeneic hematopoietic stem cell (HSC) transplantations for the treatment of hematological and nonhematological diseases have been performed using HSC from umbilical cord blood (CB). However, the wide utilization of CB as a source of HSC is limited by the low number of cells recovered and thus they need to be expanded. This article shows that placenta-derived cells represent a valuable option and can support the ex vivo expansion of HSC and hematopoietic progenitor cells. Placenta-derived cells could contribute to address the unmet need of high HSC content in CB units available for transplantation.

1 | INTRODUCTION

During the last 20 years, umbilical cord blood (CB) has become an attractive source for allogeneic hematopoietic stem cell (HSC) transplantation in addition to other sources such as bone marrow (BM) aspirates.[1-4] Indeed, CB is easily available with a low risk of graft vs host disease and a high presence of HSCs and hematopoietic progenitor cells (HPCs). However, the limited number of HSC and HPC obtained from a single CB unit remains insufficient for the complete reconstitution of BM hematopoiesis in an adult patient.[1,5] Many efforts have been made to circumvent this limitation by a double cord transplant,[6] or by the ex vivo expansion of the HSC and HPC.[7,8] As a matter of fact, a cocktail of selected cytokines relevant for their role on hematopoiesis and engraftment is nowadays used for the ex vivo expansion. Three of these, stem cell factor (SCF), thrombopoietin (TPO), and the Feline McDonough sarcoma (FMS)-like tyrosine kinase 3 ligand (Flt3-L), have been reported as important for triggering the proliferation, stemness maintenance, and improved engraftment of the ex vivo-expanded CB-HSC.[9,10] In addition, copper chelating agents[11,12] or transcriptional activators and inhibitors[13-15] have also been used to increase HSC and HPC numbers for transplantation.

Currently, the use of feeder cells in combination with various cytokine cocktails to mimic the niche microenvironment is being investigated.[1,7,8,16] Importantly, in the HSC niche, mesenchymal stromal cells (MSCs) play a pivotal role by regulating the complex molecular signaling involved in HSC homeostasis.[17-20] Nowadays, BM-derived MSC (BM-MSC) represent the elective source of MSC considering their function in the HSC niche. However, over the years, several groups reported that MSC derived from perinatal tissues, such as amniotic membrane,[21] chorionic villi,[22-24] maternal decidua *basalis*,[25,26] and from the umbilical cord (including Wharton's jelly),[27,28] can constitute an alternative feeder to BM-MSC.

During fetal development, the placenta partly supports hematopoiesis, and thus it is conceivable that it is endowed with characteristics common to the hematopoietic niche.[29-31] Moreover, perinatal tissues are considered biological waste after term delivery and are therefore widely available.

Of particular interest are MSC derived from the amniotic membrane of term placenta (hAMSC). They present a phenotype consistent with MSC from fetal membranes, as suggested by a consensus paper established during the First International Workshop on Placenta-Derived Stem Cells in 2008.[21,32] In fact, these cells are marked by the classical MSC proteins CD90, CD44, CD73, and CD105, while they lack hematopoietic markers CD45, CD34, CD14,[33] and human leukocyte antigen-antigen D-related (HLA-DR) and present the in vitro differentiation potential toward one or more lineages including osteogenic, adipogenic, chondrogenic, and vascular/endothelial.[21,34] hAMSC also have immunomodulatory activity and are proposed as treatments of inflammatory-related disorders.[35,36] Indeed, several papers reported that hAMSC transplantation triggers regeneration in animal models of liver[37] and lung fibrosis,[38] collagen-induced arthritis, inflammatory bowel disease, dextran-induced severe colitis, experimental autoimmune encephalomyelitis (EAE, an animal model for multiple sclerosis),[39] traumatic brain injury,[40] and cardiac ischemia.[41] The therapeutic effects were observed in the absence of cell engraftment in the injured tissue suggesting that hAMSC act in a paracrine manner.[35,36] This hypothesis was confirmed by studies using conditioned medium (CM) derived from hAMSC that also demonstrated beneficial effects in preclinical models of lung fibrosis,[42,43] cardiac ischemia,[44] Huntington's disease,[45] and skin wound in diabetic mice.[46]

Herein, we investigated if hAMSC could represent a valid alternative to BM-MSC as a feeder layer to support HSC and HPC ex vivo expansion. We assessed the expansion and maintenance of the most primitive long-term HSC (LT-HSC), as well as the expansion of short-term HSC (ST-HSC) that are known to rapidly reconstitute the HSC niche. We also investigated the expression of adhesion molecules involved in stemness maintenance and homeostasis of HSC. Our findings suggest that hAMSC can be a valid alternative to BM-MSC for the ex vivo expansion of the CB-HSC that could help meet the growing needs of HSC for transplants.

2 | MATERIALS AND METHODS

2.1 | Ethics statements

Amniotic membrane-derived MSC (hAMSC) (n = 11) isolated from human term placenta and CB mononuclear cells (CB-MNC) (n = 12) were obtained from healthy women after full-term vaginal delivery or caesarean section.

BM-MSC (n = 9) were obtained from BM aspirates from healthy donors.

Samples were processed after informed written consent.

2.2 | MSC isolation from human BM

BM-MSCs were isolated by plastic adherence as previously described.[47] After isolation, MNCs were then seeded at a density of 0.5×10^6 cells/cm^2 in low glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (200 mM L-Glu), and 1% penicillin and streptomycin (P/S, 10.000 U P and 10 mg/ml; all from Merck, St. Louis, Missouri).

Every 3 days, nonadherent cells were removed, and fresh complete medium was added. Cells were expanded until 80% confluency and then detached with Trypsin/EDTA solution (0.25%, Merck) and expanded until passage 2 (p2) by plating at a density of 1×10^4 cells/cm^2 in DMEM complete medium at 37°C in 5% CO_2.

The cells were cryopreserved in liquid nitrogen at the final concentration of 1×10^6 cells/mL in FBS supplemented with 10% dimethyl sulfoxide (DMSO) (Merck) until use.

2.3 | MSC isolation from human amniotic membrane

MSCs derived from the human amniotic membrane (hAMSC) were isolated from human term placentas of healthy women. Isolation, phenotype characterization, and cryopreservation were carried out as previously described.[48]

Amnion fragments were digested at 37°C for 9 minutes with 2.5 U/mL dispase (VWR, Radnor, Pennsylvania); samples were then transferred to RPMI complete medium composed of RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% P/S, and 1% L-glutamine. Enzymatic digestion continued with the treatment of the amnion fragments with 0.94 mg/mL collagenase and DNase I (both from Roche, Basel, Switzerland) for approximately 2.5 to 3 hours at 37°C. The resulting cell suspensions were centrifuged at 150*g*. The supernatant was filtered through a 100-μm cell strainer (from BD Falcon, Bedford, Massachusetts) and the cells were collected by centrifugation. Freshly isolated (p0) cells were then cryopreserved in liquid nitrogen at the final concentration of 1×10^6 cells/mL in FBS supplemented with 10% DMSO until their use.

2.4 | Analysis of BM-MSC and hAMSC phenotype

Both BM-MSC and hAMSC cell populations were analyzed by flow cytometry for the expression of canonical MSC markers[21,33,49]: CD90 (clone 5E10), CD105 (clone 266), CD73 (clone AD2), the absence of hematopoietic markers such as CD45 (clone HI30) and CD34 (clone 581/CD34), and of epithelial markers as CD324 (clone 67A4), the expression of histocompatibility markers as HLA-ABC (clone G46-2.6), HLA-DR (clone TU36), and the expression of markers involved in the maintenance of HSC stemness: nestin (clone 25/NESTIN), ICAM-1/CD54 (clone HA58), and PDGFR-β/CD140b (clone 28D4).[18,50] All antibodies were purchased from BD Biosciences (BD Biosciences, Franklin Lakes, New Jersey). Cells were kept in culture for 24 hours in StemSpan™ SFEM (Stemcell Technologies, Vancouver, Canada) medium in order to mimic the experimental coculture condition. Then, cells were harvested, counted, and transferred in micronic tubes (Micronic B.V., the Netherlands). Cells were washed with PBS and centrifuged at 450*g* for 5 minutes at 4°C. The supernatants were discarded, and cells were stained with 40 μL of eBioscience™ Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific, Massachusetts) (diluted 1:1600 in PBS) for the exclusion of dead cells according to the manufacturer instructions. Blocking was performed with immunoglobulin solution (30 μL/sample).

Cells were then stained for all the aforementioned markers at 4°C for 30 minutes. Intracellular staining for nestin was performed at 4°C for 30 minutes upon fixation and permeabilization was performed with BD Cytofix/Cytoperm buffer (BD Biosciences). Antigen expression was detected using FACSAria III (BD Biosciences) and data were analyzed with FCS express v5 software (De Novo Software, Los Angeles, California).

2.5 | Mononuclear and CD34$^+$ cell isolation from human umbilical CB

Samples were collected by umbilical CB aspiration using CB collection set (JMS Singapore Pte Ltd) and processed immediately. CB-MNCs were isolated over a Ficoll-paque density gradient medium (1.077 g/mL) (Merck) and centrifuged at 800*g* for 30 minutes. CB-MNC were then cryopreserved in liquid nitrogen at the final concentration of 20×10^6 cells/mL in FBS supplemented with 10% DMSO until their use.

CD34$^+$ HSCs were purified from total CB-MNC by positive selection using anti-CD34-coated microbeads and MACS® separation columns following manufacturer instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

To increase the purity of CD34$^+$ cells, the eluted positive fractions were enriched by a second magnetic separation in Medium Size column (MS, Miltenyi Biotec). Finally, the CD34$^+$ cell number and viability were determined by Trypan blue exclusion assay. The obtained samples of CD34$^+$ cells showed a purity > 95% evaluated by flow cytometry using FACSAria III. Data were analyzed with FCS express v5 software (De Novo Software).

2.6 | CB-CD34$^+$ cells and MSC cocultures

To analyze the effect of the two different MSC feeders on CD34-HSC *ex vivo* expansion, BM-MSC and hAMSC were seeded in 24 well plates (Corning, New York, USA) in StemSpan™SFEM (Stemcell Technologies, Vancouver, Canada) medium at two different concentrations, 40,000 and 160,000 cells in 0.5 ml per well, and allowed to adhere overnight. The day after, MSC were gamma-irradiated at 30Gy to block cell proliferation and CD34$^+$ isolated HSC (20,000 cells/ in 0.5 ml) were added. The coculture was thus performed with two different CD34$^+$-MSC ratios (1:2 and 1:8). Stem Span SFEM culture

medium was supplemented with three cytokines necessary for the expansion and overall survival of $CD34^+$ cells[51,52]: SCF, Flt-3L and TPO at the concentration of 30 ng/ml each (all from Miltenyi Biotec). Cocultures were established either in cell-to-cell contact or using transwell chambers (0.4 μm pore, polycarbonate membrane; Corning). To detect the basal effect of the cytokine cocktail on the ex vivo expansion of $CD34^+$ cells, the control condition represented by the ex vivo expansion in absence of a feeder layer was included.

Cocultures and $CD34^+$ cells cultured alone were incubated for 7 days at 37°C in humidified atmosphere and 5% CO_2. After 7 days, the mixed population composed of $CD34^+$ HSC and MSC, as well as the $CD34^+$ HSC cultured alone representative of the basal condition, was collected and cell proliferation and viability were determined by Trypan blue exclusion assay. Cells were then analyzed for their phenotype.

2.7 | Flow cytometry analysis of ex vivo-expanded CB-$CD34^+$ cells

The ex vivo-expanded $CD34^+$ cells as well as time zero (T0) freshly isolated $CD34^+$ cells seeded to perform the coculture were phenotypically analyzed for a panel of antibodies to identify and discriminate primitive $CD34^+$ cells from the most advanced progenitor.

Cells were harvested as described in paragraph 2.4 and stained with anti-CD45 (clone HI30), anti-CD45RA (clone REA562), anti-CD34 (clone581) and anti-CD38 (clone LS198-4-3), anti-CD133 (clone AC133), and anti-CD7 (clone M-T701) for lymphoid progenitors, anti-CD33 (clone P67.6) for myeloid progenitors, and CD71 (M-A712) for megakaryocyte-erythrocyte progenitors. In detail, LT-HSCs were identified as CD45RA-$CD34^+CD38^-CD133^+$, ST-HSCs as $CD45RA^-CD34^+CD38^+CD133^-$. For analysis of HPC and most committed progenitors, the gating strategy was the following: HSC/multipotent progenitors (MPPs) were identified as $CD34^+CD38^-CD45RA^-CD133^+CD7^-$; lymphoid-primed multipotent progenitors (LMPP) as $CD34^+CD38^-CD45RA^+CD133^-CD7^-$, multi lymphoid progenitors (MLP) as $CD34^+CD38^-CD45RA^+CD133^-CD7^+$; common myeloid progenitors (CMP) as $CD34^+CD38^+CD45RA^-CD7^-CD71^-$; the granulocyte-monocyte progenitors (GMP) as $CD34^+CD38^+CD45RA^+CD7^-CD33^+$ and, finally, megakaryocyte-erythrocyte progenitors (MEP) as $CD34^+CD38^+CD45R^-CD7^-CD71^+$.[53,54] All antibodies were purchased from BD Biosciences except for CD34 that was purchased from Becton Dickinson. Cells were acquired using FACSAria III and the analyzed with FCS express v5 software (De Novo Software).

2.8 | Colony forming unit (CFU) assay

The clonogenic capacity of $CD34^+$ cells expanded after 7 days of coculture with BM-MSC, hAMSC, or in the control condition was evaluated by short-term CFU assay. Cells for burst-forming units erythroid (BFU-E), colony-forming unite granulocyte-macrophage (CFU-GM), and CFU-granulocyte/macrophage/erythroid/megakariocyte (CFU-GEMM) were seeded in duplicate in methylcellulose MethoCult H4434 classic medium (Stemcell Technologies) in 35 mm petri dishes (Corning). Cells were incubated for fourteen days at 37°C in a humidified atmosphere at 5% CO_2 according to the manufacturer's instructions. After 14 days, BFU-E, CFU-GM, and multilineage colonies (GEMM) were scored in situ.

2.9 | Long-term culture-initiating cell (LTC-IC): bulk culture assay

To evaluate the capacity of ex vivo $CD34^+$ cells expanded in the presence of the two different MSC feeders, as well as in the control condition, to support long-term hematopoietic cell niche reconstitution, $CD34^+$ cells harvested from primary cocultures with BM-MSC or hAMSC were tested for LTC-IC assay. M2-10B4 cells (Stemcell Technologies) were thawed, irradiated (80 Gy), and 3×10^5 cells were seeded on collagen-coated (Merck) six-well plates (Corning) in MyeloCult H5100 medium (Stemcell Technologies) supplemented with 10^{-6} M hydrocortisone (Stemcell Technologies) and allowed to adhere overnight. The day after, 30 000 $CD34^+$ expanded cells were added to each well. Cultures were maintained for 6 weeks at 37°C in 5% CO_2,[55-57] with half-medium changes once per week. After 6 weeks, the total cell fraction composed by a mix of nonadherent and trypsinized adherent cells of each culture was harvested, counted with Trypan blue exclusion dye, and 50 000 cells were seeded in MethoCult H4435 Enriched medium (Stemcell Technologies) 35 mm dishes for the CFU assay and the total number of colonies obtained were counted after 14 days.

The frequency of LTC-IC in the starting cell population and the average number of CFU derived from each LTC-IC were calculated as previously reported.[58-60]

2.10 | Statistical analysis

The data are displayed as truncated violin plots histograms with median and quartile. The parameters were compared using one- or two-way analysis of variance. Data are representative of at least four independent experiments. Statistical analysis was performed using Prism 8 (GraphPad Software, La Jolla, California). A *P*-value lower than .05 was considered statistically significant.

3 | RESULTS

3.1 | hAMSCs and BM-MSCs present similar immunophenotype

BM-MSCs and hAMSCs were confirmed to be morphologically similar (Figure 1A). Both MSC cell populations expressed the canonical MSC

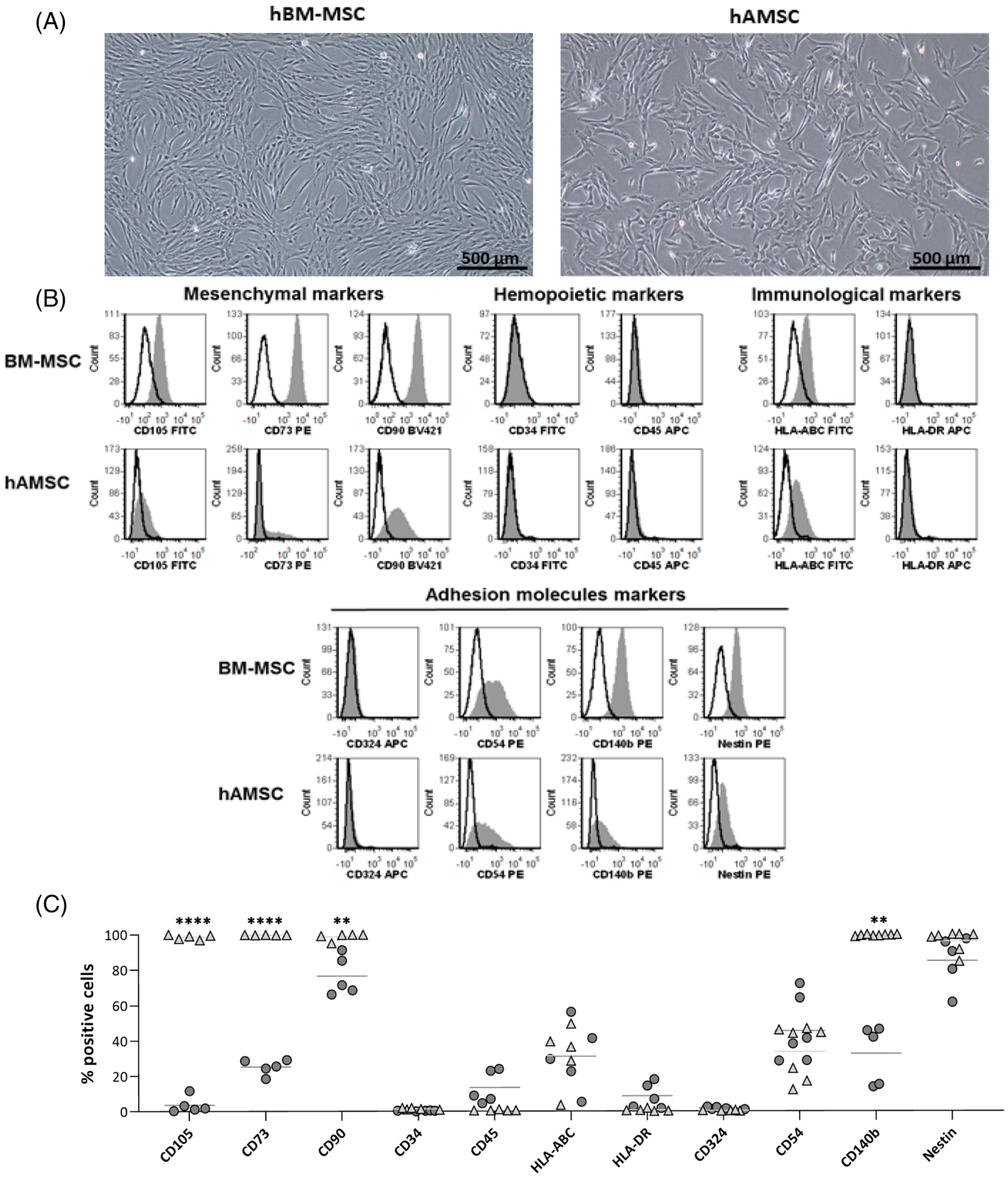

FIGURE 1 A, Bright-field microscopy was used to analyze mesenchymal stromal cell (MSC) morphology; scale bar = 500 µm. B, Immunophenotype of human bone marrow MSCs (BM-MSCs) and amniotic mesenchymal stromal cells (hAMSCs). Phenotype was analyzed by flow cytometry and in (C) the percentage of positive BM-MSCs (triangle) or hAMSCs (circle) for each marker was represented. Data are presented as mean ± SD (**$P \leq .01$, ****$P \leq .0001$) and they are representative of ≥5 different MSC donors. APC, allophycocyanin; BV-421, brilliant violet 421; FITC, fluorescein isothiocyanate; PE, phycoerythrin

markers CD90, CD105, and CD73[21,33] and had a low/absent expression of hematopoietic markers (CD45 and CD34) and of the epithelial marker CD324 (Figure 1B,C). Moreover, both BM-MSCs and hAMSCs expressed HLA-ABC, and were negative for HLA-DR. In addition, both BM-MSC and hAMSC also expressed markers essential for HSC stemness: CD54/ICAM-1 (62.61% ± 16.83 SD vs 63.4% ± 0.3 SD); nestin (83.15 ± 22.84 SD vs 63.01% ± 12.76 SD); and CD140b (96.89% ± 3.65 SD vs 48.48% ± 8.04 SD), with a significant difference observed for the expression of CD140b.

3.2 | hAMSCs support the ex vivo expansion of CB-HSC not only in contact-dependent culture but also in transwell system

The capacity of hAMSCs to support the ex vivo expansion of CB-HSC cells was evaluated in comparison to the control condition represented by the CB-CD34$^+$ cells without feeder layer. The coculture was performed at two different CD34$^+$:MSC ratios (1:2 and 1:8) in both cell-to-cell contact and transwell conditions. Data were analyzed as total number of cells counted (Figure 2A) and as fold increase (Figure 2B) obtained by the ratio between the total number of cells collected after 7 days of coculture with MSCs vs the basal control condition (CTR) (first lane Figure 2A).

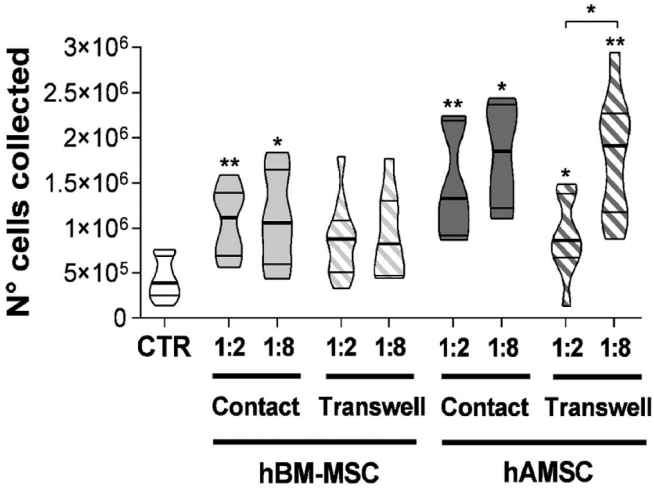

FIGURE 2 Effects of human amniotic mesenchymal stromal cells (hAMSCs) and bone marrow mesenchymal stromal cells (BM-MSCs) on cord blood hematopoietic stem cells (CB-HSC) ex vivo expansion. The cocultures were performed with HSCs and BM-MSCs or hAMSCs at two different CD34$^+$:MSC ratios (1:2 and 1:8), either in cell-to-cell contact (full) or transwell (oblique lines) setting for 7 days in serum-free medium supplemented with a cytokine cocktail composed by stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt3-L), and thrombopoietin (TPO). The control condition, indicated as CTR, is representative of the ex vivo expansion in the absence of a feeder layer. Results are expressed as total number of cells collected after 7 days of ex vivo expansion. Data are represented as mean ± SD (*$P \leq .05$, **$P \leq .01$) from ≥ 4 independent experiments

In cell-to-cell contact, both concentrations of BM-MSCs induced a significant increase of the ex vivo-expanded HSCs with a fold change of 2.39 ± 0.73 SD and 2.41 ± 1.01 SD, respectively, in comparison to the CTR (Figure 2B). Similar results were obtained for the transwell setting where there was a 2.55 ± 1.26 SD and 3.09 ± 1.31 SD fold increase compared with the CTR (Figure 2B). Notably, in cell-to-cell contact, hAMSCs were able to induce a stronger proliferation reaching a fold increase of 3.27 ± 0.99 SD and 4.23 ± 1.53 SD (1:2 and 1:8, respectively). Both ratios of hAMSCs in the transwell setting supported ex vivo expansion of CD34$^+$ cells with a fold increase of 2.98 ± 1.39 SD and 5.63 ± 2.09 SD (1:2 and 1:8, respectively) (Figure 2B).

3.3 | hAMSCs expand both short-term and long-term HSC

Different studies have correlated the proliferation rate with the differentiation state of CD34$^+$ cells.[61-63] In order to evaluate the ability of the two MSC feeders not only to favor CD34$^+$ ex vivo expansion, but also to preserve their stemness, the expansion of the primitive progenitor subpopulations, named LT-HSC (identified as CD34$^+$CD38$^-$CD133$^+$) and ST-HSC (identified as CD34$^+$CD38$^+$CD133$^-$), was evaluated by immunophenotype analysis (Figure 3).

At both CD34$^+$:MSC ratios analyzed, BM-MSCs induce a modest expansion of LT-HSC in the contact setting (2.63 ± 0.71 SD and 2.46 ± 1.03 SD for 1:2 and 1:8, respectively) compared with the CTR condition represented by CD34$^+$HSC cultured alone. Comparable results were obtained for BM-MSCs in the transwell setting (2.76 ± 1.88 SD and 2.55 ± 2.15 SD for 1:2 and 1:8 ratios, respectively). Similar findings were observed with hAMSCs that were able, in cell-to-cell contact, to stimulate the ex vivo expansion of LT-HSC (1:2 CD34$^+$:MSC ratio 3.19 ± 3.22 SD vs the CTR condition).

On the other hand, the 1:8 CD34$^+$:MSC ratio determined only a slight increase (0.75 ± 0.38 SD vs the CTR condition). At both ratios in the transwell setting, only a slight increase in the total amount of LT-HSC was observed in comparison to the CTR (2.73 ± 2.24 SD and 2.84 ± 3.29 SD fold change vs CTR for CD34$^+$:hAMSC 1:2 and 1:8, respectively) and comparable to what previously observed for the BM-MSCs at the same culture condition.

Furthermore, a higher increase of ST-HSC in the presence of BM-MSCs was observed in the contact setting (4.22 ± 1.60 SD and 7.17 ± 2.81 SD for 1:2 and 1:8, respectively), while in the transwell setting a significant increase was observed with the highest BM-MSC concentration (5.69 ± 2.64 SD and 12.79 ± 6.57 SD for 1:2 and 1:8, respectively). In addition, at the highest hAMSC concentration, there was an increase of ST-HSC, stronger than that observed when BM-MSCs were used at the same CD34$^+$:MSC ratio (33.70 ± 10.94 for the hAMSCs vs 7.17 ± 2.81 SD for BM-MSCs). These findings were confirmed also for the transwell setting (8.61 ± 5.91 SD and 43.40 ± 15.65 fold increase hAMSCs vs CTR for the 1:2 and 1:8 ratios).

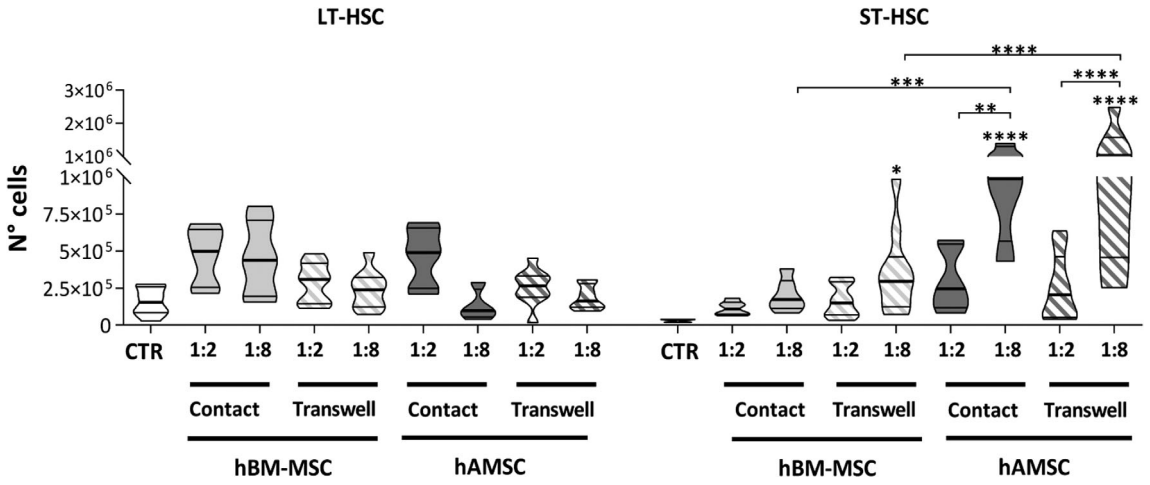

FIGURE 3 Effects of human amniotic mesenchymal stromal cells (hAMSCs) and bone marrow mesenchymal stromal cells (BM-MSCs) on hematopoietic stem cell (HSC) subpopulations: long-term (LT-HSC) and short-term (ST-HSC). The cocultures were performed with $CD34^+$ cells and BM-MSCs or hAMSCs at two different $CD34^+$:MSC ratios (1:2 and 1:8), either in cell-to-cell contact (full) or transwell (oblique lines) settings, for 7 days in serum-free medium supplemented with a cytokine cocktail (stem cell factor [SCF], FMS-like tyrosine kinase 3 ligand [Flt3-L], and thrombopoietin [TPO]). The control condition, indicated as CTR, represents the ex vivo expansion in the absence of a feeder layer. Results are expressed as total number of LT-HSC ($CD34^+CD38^-CD133^+$) and ST-HSC ($CD34^+CD38^+CD133^-$) normalized to the total amount of $CD34^+$ cells after 7 days coculture. Data are represented as mean ± SD (*$P \leq .05$, **$P \leq .01$, ***$P \leq .001$, and ****$P \leq .0001$) from ≥ 4 independent experiments

3.4 | hAMSCs support the expansion of both primitive and committed HPC subsets

The ability of the different feeder layers to foster the expansion of primitive HSC and the different HPC subpopulations was analyzed. Herein, the effect of BM-MSCs and hAMSCs on the commitment toward different primitive HPC lineages (HSC/MPP, LMPP, MLP), as well as on the most committed lineages (CMP, MEP and GMP), was analyzed (Figure 4).

No significant differences were observed among the different culture conditions in the most primitive population represented by HSC/MPP (Figure 4A). Indeed, in contact and transwell, both ratios of BM-MSCs were able to trigger an increase in the amount of HSC/MPP cells (for contact: fold increase of 3.4 ± 2.4 SD and 7.42 ± 6.19 SD 1:2 and 1:8 ratios, respectively; for transwell: 3.91 ± 2.43 SD and 5.39 ± 3.70 SD 1:2 and 1:8 ratios, respectively).

Interestingly, hAMSCs in the contact setting and at a 1:2 ratio with $CD34^+$ led to a strong increase in the number of HSC/MPP cells (11.68 ± 12.16 SD fold change vs CTR). The other three hAMSCs culture conditions: the 1:8 ratio in contact and both the transwell settings (1:2 and 1:8 ratios) lead to an increase in the primitive subpopulations with findings comparable to what observed for the BM-MSC feeder layers, at the same ratio and culture conditions (2.48 ± 1.29 SD, 4.30 ± 2.92 SD, and 6.67 ± 5.63 SD, respectively).

On the other hand, a significant increase in the LMPP subpopulation was observed in the presence of both BM-MSCs and hAMSCs, in either contact or transwell setting and at both ratios (1:2 and 1:8), compared with the CTR. At the 1:2 ratio, the effects of hAMSCs and BM-MSC were comparable (2.55 ± 0.52 SD for BM-MSCs vs 2.45 ± 0.34 SD for hAMSCs). At the 1:8 ratio, hAMSCs resulted in a reduced capability to trigger the expansion of LMPP in comparison to what observed for the BM-MSCs (2.02 ± 0.43 SD for BM-MSCs vs0.71 ± 0.29 SD for hAMSCs), while for the transwell setting the amount of ex vivo-expanded LMPP was comparable to what was previously observed for BM-MSCs (2.66 ± 1.75 SD and 2.33 ± 2.12 SD for 1:2 and 1:8 ratios, respectively for BM-MSCs vs 2.92 ± 2.25 SD and 2.8 ± 3.22 SD for the 1:2 and 1:8 ratios, respectively for hAMSCs).

No significant differences were observed in MLP compartment among the different conditions.

Importantly, concerning the effects of MSCs on the less primitive compartments (Figure 4B), hAMSCs were able to significantly induce the ex vivo expansion of committed HPC toward the CMP or GMP compartments and, to a lesser extent, the MEP compartment. In particular, a fold increase of 4.53 ± 1.55 SD and 7.54 ± 2.15 SD for both $CD34^+$:MSC ratios in the contact setting was observed in the CMP compartment, and similar findings were observed in the transwell setting (5.77 ± 2.76 SD and 13.11 ± 8.54 SD for 1:2 and 1:8 BM-MSCs ratios, respectively, in comparison to the CTR). hAMSCs at both ratios were able to lead to a strong increase of 12.01 ± 5.63 SD and 22.03 ± 12.50 SD for 1.2 and 1:8 ratios, respectively, vs the CTR for the contact setting. This was also confirmed for the transwell setting where a stronger increase was observed for the higher $CD34^+$: MSC ratio (11.30 ± 7.22 and 34.25 ± 17.92, respectively, for 1:2 and 1:8 ratios).

Moreover, BM-MSCs and most of all hAMSCs were able to increase the MEP compartment, at both ratios and in both contact and transwell settings (6.05 ± 1.41 SD and 11.83 ± 3.31 SD for BM-MSCs vs 16.09 ± 6.24 and 21.90 ± 6.72 SD for hAMSC in contact setting). The transwell setting triggered a stronger increase for both feeder layers and once again the highest increase was obtained when

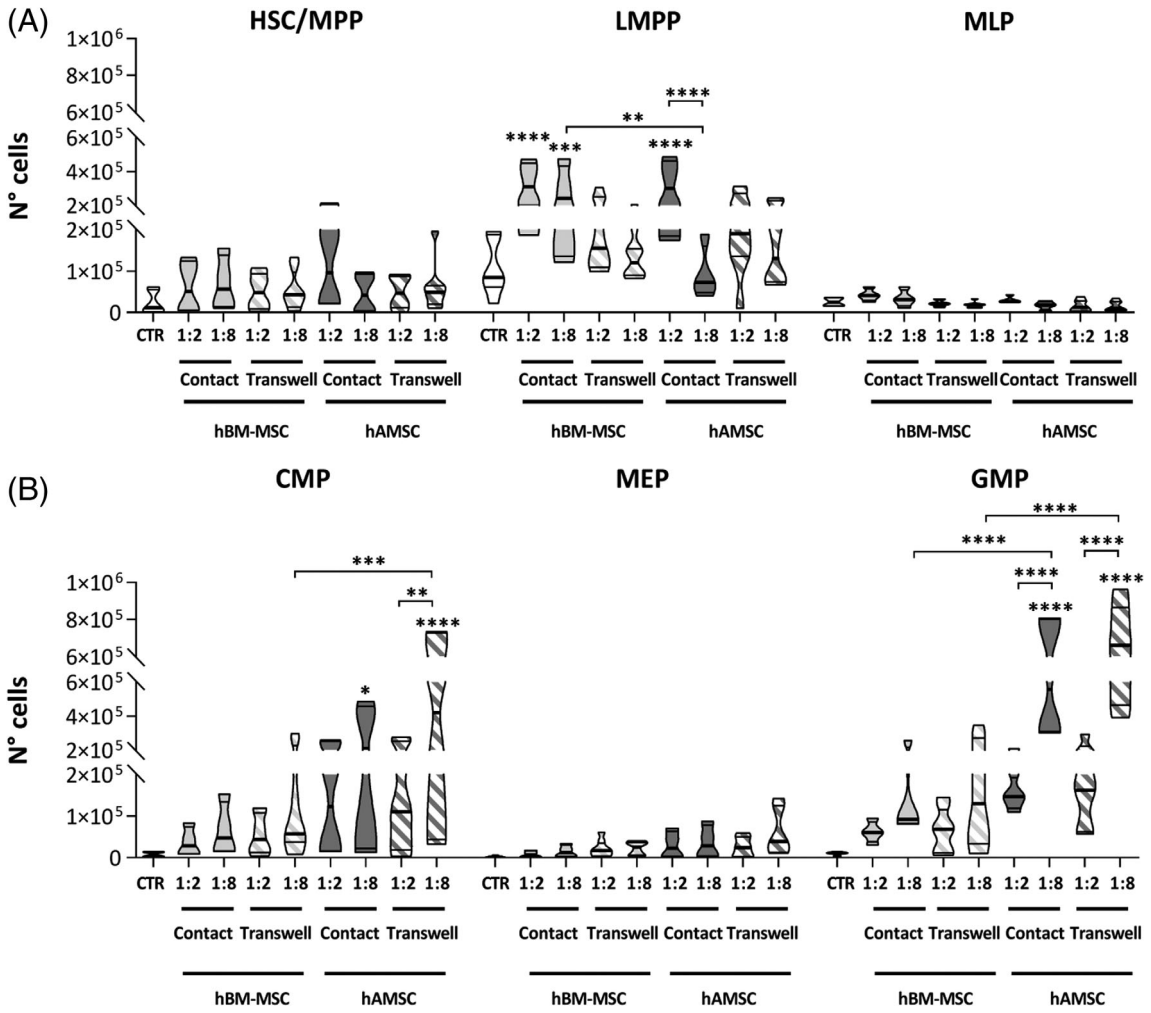

FIGURE 4 Effects of human amniotic mesenchymal stromal cells (hAMSCs) and bone marrow mesenchymal stromal cells (BM-MSCs) on HSC and hematopoietic progenitor cell (HPC) subpopulations. The cocultures were performed with $CD34^+$ cells and BM-MSCs or hAMSCs at two different $CD34^+$:MSC ratios (1:2 and 1:8, either in cell-to-cell contact (full) or in transwell (oblique lines) setting for 7 days in serum-free medium supplemented with a cytokine cocktail (stem cell factor [SCF], FMS-like tyrosine kinase 3 ligand [Flt3-L], and thrombopoietin [TPO]). The control condition, indicated as CTR, is representative of the ex vivo expansion in the absence of a feeder layer. MSC were analyzed for their effects on the commitment toward (A) different primitive HPC lineages (HSC/MPP, LMPP, MLP) and (B) on committed lineages (CMP, MEP, and GMP). The results are described as fold increase obtained by the ratio between the total number of cells collected after 7 days of coculture with MSCs vs the control condition (CTR). Data are represented as mean ± SD (*$P \leq .05$, **$P \leq .01$, ***$P \leq .001$, and ****$P \leq .0001$) from ≥ 4 independent experiments. CMP, common myeloid progenitor; GMP, granulocytic monocytic progenitor; HSC, hematopoietic stem cell; LMPP, lymphoid-primed MPP; MEP, megakaryocyte–erythrocyte progenitor; MLP, multilymphoid progenitor; MPP, multipotent progenitor

the coculture was performed with hAMSCs (12.53 ± 13.58 SD and 17.90 ± 19.67 SD for BM-MSCs vs7.69 ± 2.54 SD and 32.94 ± 21.53 SD for hAMSCs).

Finally, in the GMP compartment, both MSC, but once again most of all hAMSCs, were able to strongly induce the expansion and polarization toward this progenitor subset. Indeed, for both settings and especially at the highest $CD34^+$:MSC ratio, hAMSCs significantly induced the ex vivo expansion of GMP cells in comparison to the $CD34^+$ cells expanded using the BM-MSCs feeder layer (13.05 ± 1.4 SD and 13.97 ± 9.47 SD for 1:2 ratio with hAMSCs in contact and transwell settings, respectively, vs 5.14 ± 1.39 SD and 5.96 ± 4.01 SD for BM-MSC in contact and transwell settings, respectively). These differences were significant at the 1:8 ratio and for both settings (12.17 ± 9.86 SD and 13.70 ± 9.45 SD for BM-MSCS vs 47.84 ± 23.69 SD and 57.61 ± 19.25 for hAMSCs).

These findings confirm those in Figure 3, where hAMSCs induced a stronger increase in the ST-HSC subset in comparison to the most primitive LT-HSC.

3.5 | hAMSCs support the clonogenic ability of CB progenitor cells

A CFU assay was performed in order to evaluate the hematopoietic clonogenic ability of the ex vivo-expanded CB-HPC in different culture conditions.

Regardless of the feeder layer, an increase in the number of total colonies was observed in comparison to the CTR. In particular, a fold increase of approximately 2.22 ± 0.74 SD in the number of colonies was observed after coculture with BM-MSCs at the 1:2 $CD34^+$:MSC ratio, while a slight increase was appreciated at the 1:8 ratio (2.17 ± 0.96 SD). On the other hand, a significant increase was observed in the transwell setting (fold increase 3.09 ± 4.07 SD and 3.23 ± 4.30 SD for 1:2 and 1:8 ratios, respectively, in comparison to the CTR). Moreover, similar results were obtained with the hAMSCs feeder layer (2.68 ± 0.55 SD and 1.8 ± 0.32 SD for the 1:2 and 1:8 $CD34^+$:MSC ratio in contact setting). Similar to that observed from the coculture with the BM-MSCs feeder, hAMSCs were also able to induce a significant increase in the transwell setting (2.81 ± 4.42 SD and 2.8 ± 4.23 SD for 1:2 and 1:8 ratios, respectively) (Figure 5B).

Importantly, both BM-MSCs and hAMSCs were able to generate the most primitive CFUs—granulocyte, erythrocyte, macrophage, and megakaryocyte (also named CFU-GEMM).

In particular, a stronger increase was observed when the coculture was performed with BM-MSCs in comparison to what was observed with hAMSCs at both ratios (1.25 ± 0.58 SD and 0.83 ± 0.58 for the BM-MSCs in contact setting vs 0.83 ± 0.58 SD and 0.63 ± 0.50 for 1:2 and 1:8 ratios with hAMSCs in contact setting). Moreover, a stronger expansion in the number of CFU-GEMM by BM-MSCs, compared with hAMSCs, was also observed in the transwell setting (4 ± 0.55 SD and 3.75 ± 0.84 SD times higher for 1:2 and 1:8 ratios vs 3.75 ± 1.05 and 3.75 ± 0.84 for 1:2 and 1:8 ratios, respectively, with hAMSCs). Furthermore, similar results were obtained also for BFU-E, with a comparable amount was obtained regardless of the feeder layers used (Figure 5C).

Finally, previous observations obtained from the phenotype analysis of the different progenitor subsets were confirmed. Indeed, a specific commitment induced by the hAMSCs toward the GMP subset was previously observed (Figure 4, lower panel). These findings were also confirmed with this functional assay. Importantly, both feeders at both ratios in the contact setting were able to induce a significant increase in the total number of CFU-GM colonies (2.56 ± 4.03, 2.60 ± 4.51 for BM-MSCs vs 3.76 ± 11.24 and 2.10 ± 7.80 for hAMSCs). Similar findings were observed for the transwell setting, where both BM-MSCs and hAMSCs were able to induce a significant expansion in the total amount of CFU-GM (3.67 ± 9.29, 3.59 ± 21.30 for BM-MSCsvs3.42 ± 17.89 and 3.94 ± 16.54 for hAMSCs).

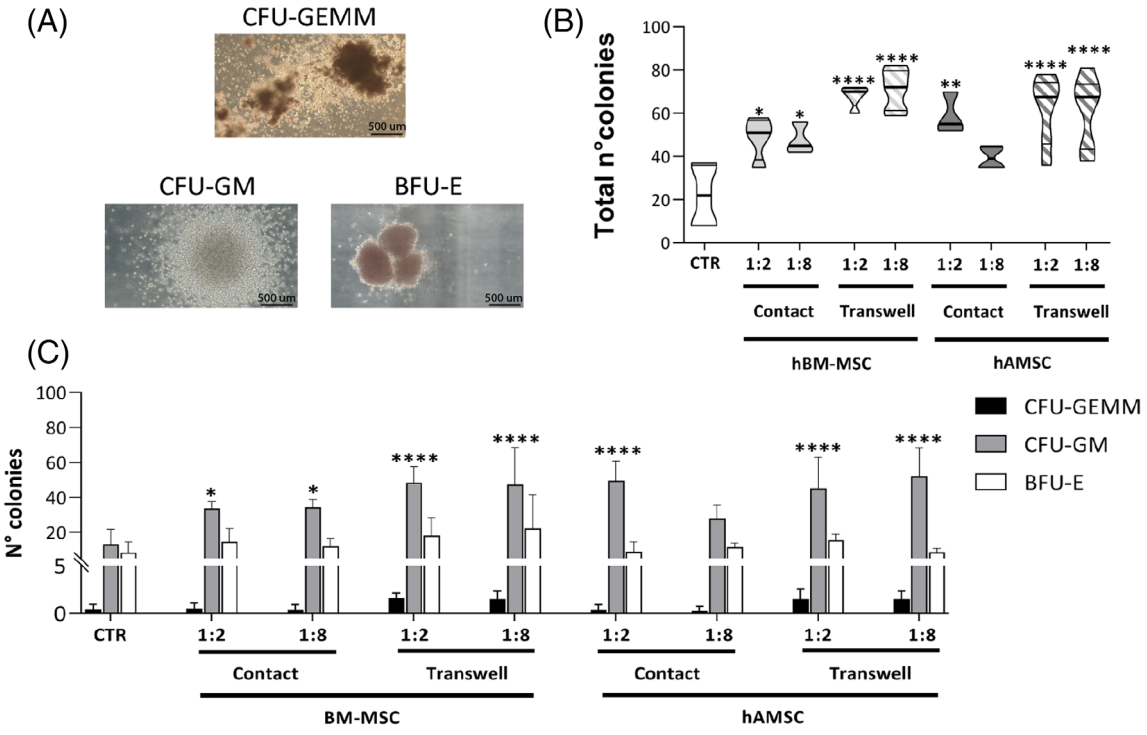

FIGURE 5 Effects of hAMSCs and BM-MSCs on the clonogenic capacity of cord blood $CD34^+$ cells (CB-$CD34^+$ cells). $CD34^+$ cells were ex vivo-expanded for 7 days in different culture conditions in MethoCult™ medium. The total number and different types of colonies obtained were counted after 14 days. A, Bright-field microscopy was used to identify colony subtypes; scale bar = 500 μm. B, Graph represents the number of colony forming unit with the different cell culture conditions. C, Graph represents the number of each colony forming units after 14 days of culture in MethoCult™ medium. Data are represented as mean ± SD (*$P \leq .05$, and ****$P \leq .0001$) from ≥ 4 independent experiments. BFU-E, burst forming unit – erythroid; BM-MSCS, human bone marrow mesenchymal stromal cells; CFU-GEMM, colony forming unit—granulocyte, erythrocyte, macrophage, megakaryocyte; CFU-GM, colony forming unit—granulocytes, macrophages; CTR, control or no feeder layer; hAMSCs, human amniotic mesenchymal stromal cells

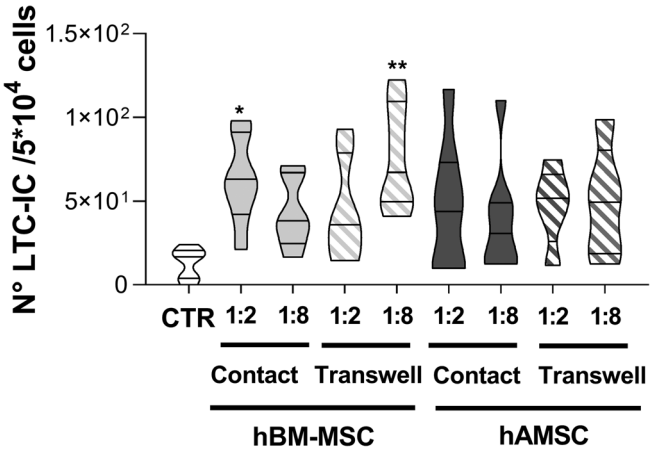

FIGURE 6 Effects of hAMSCs and BM-MSCs on long-term cultures initiating cells (LTC-IC). $CD34^+$ cells ex vivo expanded on BM-MSCs or hAMSC or without feeder layer (control) at $CD34^+$:MSC ratios (1:2 and 1:8) were seeded on irradiated M2-10B4 cells for 6 weeks in MyeloCult H5100™ medium supplemented with 10^{-6} M hydrocortisone with half-medium changes once per week. After 6 weeks, the total cell fraction of each culture was harvested and 50 000 cells were seeded in MethoCult H4435 Enriched™ medium to perform the CFU assay. The total number of colonies obtained was counted after 14 days. The graph represents the number of LTC-IC with the different cell culture conditions. Data are represented as mean ± SD (*$P \leq 0.05$, **$P \leq .01$) from ≥5 independent experiments. BM-MSCs, human bone marrow mesenchymal stromal cells; CTR, control or no feeder layer; hAMSCs, human amniotic mesenchymal stromal cells

3.6 | hAMSCs support LTC-ICs

We analyzed the capacity of BM-MSCs and hAMSCs to support LTC-ICs. We observed an increase of the total number of LTC-IC in the presence of both feeder layers, in comparison to the CTR (Figure 6).

Indeed, BM-MSCs induced a significant increase in the total number of LTC-IC for the 1:2 $CD34^+$:MSC ratio, while the increase was weaker for the 1:8 ratio (12.84 ±16.96 SD for the 1:2 vs 10.12 ±14.45 SD for the 1:8 $CD34^+$: MSC ratio). In the transwell setting, a stronger increase was observed for the 1:8 $CD34^+$:MSC ratio while no significant differences were observed for the 1:2 $CD34^+$: MSC ratio in comparison to the CTR (fold increase of 5.93 ± 5.07 SD and 21.79 ± 31.98 for 1:2 and 1:8 ratios, respectively).

Similarly, hAMSCs showed a trend for a higher amount of LTC-IC than CTR, but the increase was not statistically significant. Indeed, at the 1:2 $CD34^+$:MSC ratio, hAMSCs cultured in contact with $CD34^+$cells were able to induce an increase in LTC-IC in comparison to the CTR (fold increase 6.24 ± 4.06 SD). On the other hand, a slight increase was appreciable in comparison to the CTR, as previously reported for the BM-MSC, for the 1:8 $CD34^+$:MSC ratio in contact setting (fold increase 12.50 ± 16.68 SD time the control condition). Importantly, for the transwell setting, hAMSC presented the capacity to trigger the expansion of LTC-IC with results comparable to those obtained with the hAMSC feeder in the contact setting (2.67 ± 0.62 SD and 6.51± 4.08 SD in transwell setting for 1:2 and 1:8 ratios, respectively).

4 | DISCUSSION

The ex vivo expansion of CB-HSCs is required in order to improve the efficacy of transplantation in adult patients.[7,8] To date, CB-HSCs are expanded either through coculture systems with supportive feeders such as BM-MSCs, a major component of the hematopoietic niche in BM, or through the use of growth factor cocktails.[1,7,8,16] BM-MSCs currently represent the gold standard condition for ex vivo expansion of HSCs, but their procurement presents a number of obstacles such as invasiveness and risks for the donor. In the last decade, several studies have shown how MSC isolated from other sources can be a viable alternative.[64-67]

Here, we demonstrate that both BM-MSC and hAMSCs can (a) trigger the ex vivo expansion of CB-HSC in both contact as well as in transwell system, (b) prompt the expansion of both LT-HSC as well as ST-HSC, (c) favor the expansion of different HPC progenitor subsets, while maintaining and fostering the expansion of the most primitive HSC/MPP subsets, (d) maintain the capacity to form CFU colonies and lastly, (e) possess the ability to trigger a slight increase in the number of LTC-ICs.

We confirmed the similar immunophenotype of BM-MSCs and hAMSCs. Concerning CD105, a typical MSC marker, we confirmed its reduced expression by hAMSC at low cell culture passages.[49]

Furthermore, both BM-MSCs and hAMSCs expressed CD54 (Intercellular Adhesion Molecule 1, ICAM-1),[66,68] a receptor involved in the repopulation and homing of the transplanted HSC.[18] As a matter of fact, CD54 (ICAM-1)-deficient mice ($CD54^{-/-}$) have an impairment of quiescence and repopulation capability of the HSC niche.[69] We also confirm the variable expression of PDGFR-β, also known as CD140b, in hAMSC, as previously reported.[70,71] The expression of CD140b has been shown to correlate with enhanced survival and expansion after transplantation.[72] Finally, we report that hAMSC highly expressed nestin, which is associated with HSC quiescence and maintenance in the BM niche.[73-75]

Interestingly, although some previous studies showed the importance of cell-to-cell contact for the maintenance of stemness,[64-67] we observed ex vivo expansion also in the transwell setting condition, suggesting that both MSC can release bioactive molecules that can trigger the ex vivo expansion of HSC cells and its progenitors. These findings support the study by Fong and colleagues that reported how CM of Wharton's jelly MSC was able to expand CB-$CD34^+$ cells better than the use of the cells in contact setting.[76] On the other hand, when we analyzed the expansion of the most primitive HSC subsets, the long-term reconstituting HSC (LT-HSC) and the ST-HSC, we observed a different capacity among the two MSC feeder layers in favoring the expansion of one subpopulation instead of the other. These subpopulations are representative of two different stages of differentiation that can be distinguished both phenotypically and functionally. The most primitive, the LT-HSC, is characterized by the

greatest self-renewal capacity and gives rise to all hematopoietic lineages throughout life. ST-HSC presents instead a reduced self-renewal potential generating all the hematopoietic lineages for a short period.[77,78] hAMSC were able to favor the expansion ST-HSC, and a strong increase was observed, in particular, in the transwell system. These findings were also confirmed by FACS analysis on different HSC and HPC subsets, where we did not observe any significant differences in the expansion of the most primitive HPC progenitors (HSC/MPP, LMPP, and MLP), whereas relevant differences were appreciable for the most committed CMP and GMP progenitors.[53,54] The large variability observed in the hAMSC transwell system at the highest ratio ($CD34^+$-HSC:MSC, 1:8) may be related to the high number of cells used, which could ultimately impact cell viability and consequently also the factors secreted in the transwell setting used. When we evaluated the hematopoietic clonogenic ability of the ex vivo-expanded CB HPC in the different culture conditions, we observed that both BM-MSC and hAMSC feeders were able to generate the different subtypes of colonies. The strong increase in the CFU-GM subset is in line with what was previously reported,[57,79] although, in contrast to what has been reported by others, we did not observe any significant differences in the amount of BFU-E.[80]

Lastly, we studied and compared the capacity of the two feeders to ex vivo-expanded LTC-IC able to produce myeloid progenitors for at least 5 weeks.[58,60] This long-lasting population is relevant because it repopulates the HSC niche upon transplantation.[58,60] Here, we report that both BM-MSC and hAMSC were able to support the maintenance and expansion of primitive LTC-IC. We observed a strong proliferative burst of $CD34^+$HSC obtained in the presence of hAMSC, while stemness properties were maintained, as confirmed by both the immune phenotype and the clonogenic ability. Some of the differences observed in the phenotype and in the capability of hAMSC and BM-MSC to support the ex vivo expansion of $CD34^+$ can be partly explained by the difference in the cell passage. Indeed, while hAMSCs were used at passage zero, this was not possible for BM-MSCs since the isolation process for these cells requires selection by plastic adherence.[33] Furthermore, although for hAMSC it is possible to obtain a high amount of cells directly from the isolation process, this is not possible for the BM-MSCs that require subsequent steps of in vitro expansion.

5 | CONCLUSION

The present study provides a detailed comparison of hAMSCs with the gold standard feeder represented by BM-MSCs and reports the capacity of hAMSCs to trigger the ex vivo expansion of CB-HSC, while maintaining their stemness properties as well as the capacity to differentiate, originating the different HPC progenitors. Taken together, the results suggest that the hAMSC feeder could putatively be able to reconstitute the HSC repertoire and highlight the potential application of the hAMSCs as a new alternative feeder to BM-MSCs.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

V.O., A.P.: execution of experiments, data analysis and review, manuscript writing; E.V., P.B.S., P.R.: execution of experiments, data analysis; L.D., M.M.: execution of experiments, data analysis and review; L.T.: conception and design, final approval of manuscript; A.R.S.: data analysis and review, manuscript writing; O.P.: conception and design, financial support, final approval of manuscript.

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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