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Human adipose stromal cells expanded in human serum promote engraftment of human peripheral blood hematopoietic stem cells in NOD/SCID mice

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Abstract

Human mesenchymal stem cells (hMSC), that have been reported to be present in bone marrow, adipose tissues, dermis, muscles, and peripheral blood, have the potential to differentiate along different lineages including those forming bone, cartilage, fat, muscle, and neuron. Therefore, hMSC are attractive candidates for cell and gene therapy. The optimal conditions for hMSC expansion require medium supplemented with fetal bovine serum (FBS). Some forms of cell therapy will involve multiple doses, raising a concern over immunological reactions caused by medium-derived FBS proteins. In this study, we cultured human adipose stromal cells (hADSC) and bone marrow stroma cells (HBMSC) in human serum (HS) during their isolation and expansion, and demonstrated that they maintain their proliferative capacity and ability for multilineage differentiation and promote engraftment of peripheral blood-derived CD34(+) cells mobilized from bone marrow in NOD/SCID mice. Our results indicate that hADSC and hBMSC cultured in HS can be used for clinical trials of cell and gene therapies, including promotion of engraftment after allogeneic HSC transplantation.

Keywords: Mesenchymal stem cells; Bone marrow stromal cells; Adipose tissue-derived stromal cells; Human serum; Engraftment of hematopoietic stem cells

Adult mesenchymal stem cells (hMSC) can be obtained from human bone marrow, adipose tissue, and muscle [1], and are expandable in culture [2,3], and can be genetically modified by viral and nonviral methods [4,5]. hMSC have a multipotent population of cells capable of differentiating into a number of mesodermal lineages; adipocytes, osteoblasts, and other mesodermal pathways [6,7]. Bone marrow stromal cells also support the proliferation and differentiation of hematopoietic stem cells (HSC) [8]. Adult stem cells may differentiate into tissues that during normal embryonic development would arise from a different germ layer. For example, bone marrow-derived mesenchymal stem cells may differentiate into neural tissue, which is derived from

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embryonic ectoderm [9,10]. The usefulness of hMSC administration has been demonstrated for the treatment of some genetic diseases such as osteogenesis imperfecta (brittle bone disease) [11,12], metachromatic leukodystrophy, and Hurler syndrome [13], and for hematopoietic recovery following high-dose chemotherapy in cancer patients [14]. Therefore, several characteristics of hMSC make them potentially useful for cell and gene therapy [15].

hMSC expanded in fetal bovine serum (FBS) have been used for all reported clinical trials [11–14]. hMSC themselves are not highly immunogenic [16–19]. However, residual FBS in hMSC is likely to generate immune responses, particularly in patients that repeated administrations are required. In fact, anaphylactic reactions have been noted in several patients who received repeated administrations of dendritic cells or lymphocytes cultured in FBS [20–22]. Therefore, FDA requires reduction of FBS exposure for cell culture and extensive washing before administration to patients.

Allogeneic stem cell transplantation (SCT) is being used increasingly in the treatment of hematologic and nonhematologic diseases of neoplastic and nonneoplastic origin. The transplant procedure involves infusion of HSC into the circulation, using the capacity of these cells to home to the marrow and to dock at specific sites in the marrow microenvironment [23]. Maintenance of hematopoiesis depends on the self-renewal and multilineage differentiation capacity of HSC that is thought to be regulated and controlled by the bone marrow microenvironment [24]. Marrow stroma is composed of a heterogeneous population of cells, including reticular endothelial cells, fibroblasts, adipocytes, and osteogenic precursor cells, which provide growth factors, cell-to-cell interactions, and matrix proteins [25–27]. MSC could be used either to replace host cells in the marrow microenvironment that have been damaged by chemotherapy or irradiation, or as vehicles for gene therapy. In fact, coinfusion of bone marrow-derived MSC with HSC has been reported to increase frequency and levels of HSC engraftment [28,29]. However, availability of autologous bone marrow can be limited in patients with hematologic malignancies.

The studies for replacing FBS with human serum (HS) in hMSC culture have been reported [30–33]. Kuznetsove et al. [32] reported that culture of hMSC in HS decreased their proliferation rate and differentiation potentials. Other reports showed that human serum (HS) can support short-term growth and increased spontaneous osteogenic differentiation of MSC [30,34].

Zuk et al. [35] reported that MSC from adipose tissue were cultured and differentiated in vitro into adipogenic, chondrogenic, myogenic, and osteogenic cells under appropriate culture conditions. A recent study has shown that adipose-derived-stromal cells (ADSC) can be induced to expression of neural cells in vitro [36]. We have reported that adipose tissue also contains multipotent progenitor cells, and that they have similar characteristics with bone marrow stromal cells (BMSC) in vitro and in vivo [37–39]. Therefore, human adipose tissue presents an alternative source of multipotent stromal cells.

In this study, we established the culture condition of hMSC without exposure to FBS while maintaining the proliferation and differentiation capacity necessary to generate clinically relevant numbers of cells, and examined their effects on engraftment of HSC in NOD/SCID mice to determine whether hMSC grown in HS still maintain their characteristics in vivo.

Materials and methods

Isolation and culture of stem cells hADSC and hBMSC. After informed consent, leftover materials (heparinized bone marrow cells and adipose tissues) were obtained from eight individuals undergoing total hip arthroplasty and elective plastic surgery (ages 16–54 years). hBMSC and hADSC were isolated according to the methods described in the previous studies [37,38]. Isolated cells were cultured at 37 °C/5% CO_2 in α-MEM containing 10% FBS. One week later, when the monolayer of adherent cells had reached confluence, cells were trypsinized (0.25% trypsin; Sigma) and resuspended in MD media (60% DMEM-LG (Life Technologies, Grand Island, NY), 40% MCDB-201 (Sigma) with 1× insulin–transferrin–selenium, 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen, USA), and 10 ng/mL hEGF (Daewoong Pharmaceuticals, Korea) on fibronectin (Sigma) with 5% FBS at a concentration of 2000 cells/cm^2. Before using cells for experiment, cells were cryopreserved in expansion media containing 25% HS and 10% DMSO before use in experiment.

To prepare HS, whole blood was taken from each of four consenting donors. The protocol was approved by an Institutional Review Board. The blood was recovered in the absence of anti-coagulants and allowed to clot for 4 h at room temperature. The serum was aspirated from the clot and centrifuged at 500*g* for 20 min. The supernatant was then centrifuged for a further 20 min at 2000*g*. The cleared serum was stored at −80 °C. To culture and expand cells in HS, isolated cells from collagenase treatment in adipose tissue or from Ficoll centrifugation in bone marrow were plated in α-MEM containing 10% HS. When cells reached a confluent state, cells were plated at a density of 2000 cells/cm^2 in MD or MF media containing 10% HS. In MF media, 40% MCDB-201 in MD media was replaced with 40% F-12. Cells were cryopreserved in expansion media containing 25% HS and 10% DMSO before use in experiment. To obtain sufficient amount of HS for experiment, two samples were combined to two lots. The effects of two different lots of HS were tested for each MSC sample.

Adipogenic and osteogenic differentiation. Adipogenic differentiation was induced by culturing hMSC for 2 weeks in adipogenic medium (10% FBS or HS, 1 μM dexamethasone, 100 μg/mL of 3-isobutyl-1-methylxanthine, 5 μg/mL insulin, and 60 μM indomethacin in α-MEM) and assessed using an Oil Red O stain as an indicator of intracellular lipid accumulation. In order to obtain quantitative data, 1 ml of isopropyl alcohol was added to the stained culture dish and optical density was measured at 510 nm by a spectrophotometer.

Osteogenic differentiation was induced by culturing hMSC for a minimum of 2 weeks in osteogenic medium (10% FBS or HS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid in α-MEM) and examined for extracellular matrix calcification by alizarin red stain. Osteogenic differentiation was quantified by measurement of alizarin red-stained area and density in six-well dishes by

using an image analysis program (Image Gauge ver 3.1, Fuji, Japan). Two different lots of human serum were used at each experiment and experiments were repeated with four different donors. No significant differences between the lots of each serum were found.

Measurement of population proliferation. hMSC were harvested at 90% confluence and counted. After plating the constant number of cells (10^3 cells) to a 12-well plate, cells were enumerated at each passage under hemocytometer.

Isolation of mononuclear cells from peripheral blood. In four donors who agreed to donate peripheral-blood stem cells (PBSC) during bone marrow transplantation, PBSC after receiving informed consent according to a protocol approved by the Hospital's Ethics Committee were mobilized by administration of 10 μg/kg of granulocyte colony-stimulating factor (G-CSF) daily for 5 days. After separation of peripheral blood over Ficoll–Isopaque (1.077 g/mL), low-density cells were washed in Media199 supplemented with penicillin (20 U/mL), streptomycin (20 μg/mL). The number of CD34+ stem cells in collected blood was determined by flow cytometry using CD34 antibody (Becton–Dickinson, Parsippany, NJ). Cells were cryopreserved. Cell viability of thawed cells was assayed by trypan blue dye exclusion before transplantation.

Transplantation and detection of human cells. NOD/SCID mice were sublethally irradiated (250 cGy) and transplanted within 72 h with PBSC containing 5×10^5 viable CD34(+) cells in the absence or presence of 10^6 allogeneic hMSC by intravenous injection into a lateral tail vein. hADSC or hBMSC alone without PBSC were also transplanted. No information with respect to the level of mismatch between PBSCs and MSC was available. Six weeks after transplantation, mice were killed by carbon dioxide inhalation. BM was collected by flushing both femurs with RPMI medium. Blood, obtained by cardiac puncture, was collected in tubes containing heparin and depleted of red blood cells [incubation for 10 min in NH_4Cl (8.4 g/L)/K_2CO_3 (1 g/L) buffer at 4 °C]. Human cell engraftment was detected by flow cytometry, using PE-conjugated anti-human CD45, CD33, and CD19 antibodies (Becton–Dickinson, Parsippany, NJ), and PE-conjugated isotype control antibodies (Becton–Dickinson, Parsippany, NJ). Percent human engraftment was calculated after subtraction of the background detected in control mice and 0.5% was designated as the lower threshold for unequivocal human engraftment.

Statistical analysis. Student's t test was used for statistical analysis and $p < 0.05$ was taken as significant. Differences in engraftment percentages were calculated using Fisher's exact test. Differences in engraftment percentages were calculated using the Kruskal–Wallis test. A value of $p < 0.05$ was considered statistically significant.

Results

Culture of hMSC using HS

In the previous study, we showed that MD media are superior to α-MEM media in terms of proliferation and differentiation of hMSC [38]. In a preliminary study, we observed that the proliferation of hMSC is the best in MD media containing 5% FBS. To examine if HS replaced FBS in MD media and what the optimal concentration of HS is, we examined the effect of HS (2–20%) on subsequent cultivation of hMSC that were expanded in MD containing 5% FBS (Fig. 1). After passaging cells twice in various concentrations of HS, the identical number of cells (2000 cells/cm^2/well in a 12-well plate) was plated and the cell numbers were counted at 4th day after plating. Ten percent of HS provides comparable levels of proliferation when compared with that in 5% FBS (Fig. 1). Therefore, we used 10% HS for further studies.

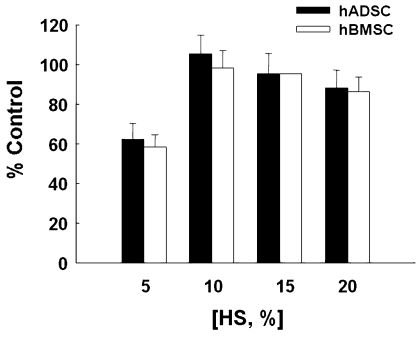


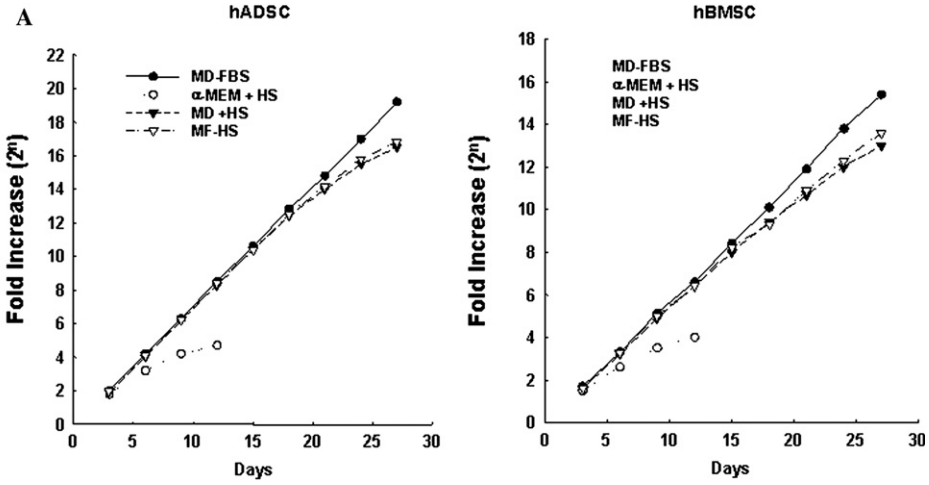
Fig. 1. Effect of HS on hMSC proliferation. In MD-FBS media, FBS was replaced with various concentrations of HS, and growth of hMSC expanded in MD-FBS was determined according to the method described in the Materials and methods. Proliferation was determined at three different MSC samples and two different lots of human serum were tested for each sample. Data are represent means ± SEM.

Then, we determined whether HS media can expand hMSC from the 1st isolation step. To perform this experiment, bone marrow mononuclear cells isolated by Ficoll–Hypaque centrifugation and adipose tissue cells isolated by collagenase treatment were directly plated in α-MEM containing 10% HS. For comparison, a half of cells were plated in α-MEM containing 10% FBS. When cells reached confluent state after plating, hMSC were subsequently cultured by MD media containing 10% HS or 5% FBS or α-MEM containing 10% HS. We also used MF media in which MCDB-201 of MD media was replaced by F-12. During subsequent cultivation, cell growth was determined. In α-MEM-HS media, their proliferation was retarded after several passages. In contrast, MSC cultured in MD or MF containing HS showed comparable growth rates with those in FBS in MD (Fig. 2A). The morphological phenotype of hMSC of 3rd passage in MD-HS or MF-HS was similar to cells grown in MD-FBS or MF-FBS (Fig. 2B). Therefore, we used MF-HS media for further experiment. We tested five different HS samples for hMSC culture and could not find any significant differences in proliferation and differentiation of cells (data not shown).

Differentiation assays

We determined whether hMSC cultured in HS can be differentiated into adipocytes and osteoblasts as readily as hMSC cultured in FBS (Fig. 3). For both osteogenesis and adipogenesis, quantitation of differentiation in four different samples demonstrated similar levels of differentiation in the MF-HS cultures and in the MF-FBS at 1st and 5th passage samples.

② A



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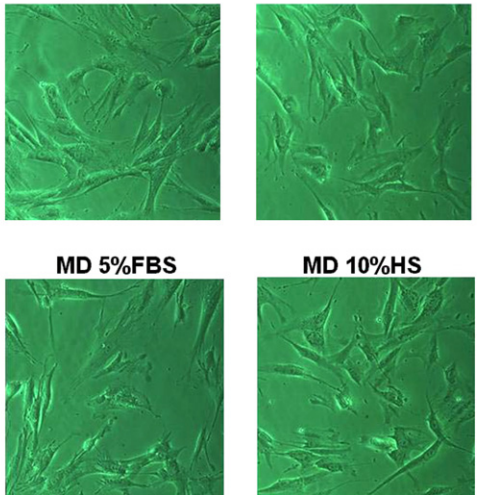

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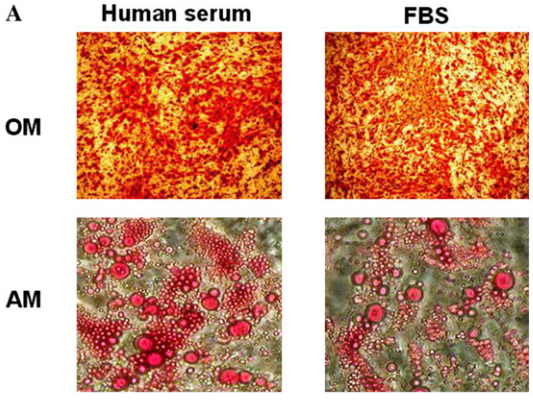

B

	hBMSC				hADSC			
	+FBS		+HS		+FBS		+HS	
	passage 1	Passage 5	Passage 1	passage 5	passage 1	passage 5	passage 1	passage 5
Adipogenic differentiation (OD/well in 12 well plate)	1.28	1.34	1.32	1.23	1.58	1.63	1.45	1.54
Osteogenic differentiation (signal intensity after 2 wks of differentiation)	100	97.3	102.7	99.8	100	99.3	96.3	100.8

Engraftment in NOD/SCID mice 6 weeks after cotransplantation of PBSC and hMSC

To determine whether hMSC expanded in HS-containing media supported engraftment and survival of human HSC in vivo, we infused human PBSC containing 5×10^5 viable CD34(+) cells with or without 1×10^6 unrelated hMSC into sublethally irradiated NOD/SCID mice. In mice receiving PBSC alone, only one out of 6 mice had detectable (>0.5%), but low, levels (<0.6%) of human engraftment after infusion. In contrast, 5 out of 6 mice had human engraftment after coinfusion of PBSC plus 1×10^6 unrelated hADSC and after coinfusion of PBSC plus 1×10^6 unrelated hBMSC ($p < 0.05$) (Fig. 4).

The level of human CD45 in the bone marrow and peripheral blood of NOD/SCID mice was also significantly higher in mice coinfused with hADSC and hBMSC ($3.64 \pm 2.30\%$ and $3.11 \pm 2.47\%$ vs $0.17 \pm 0.21\%$ in bone marrow; in peripheral blood) compared to PBSC alone (Fig. 5). The transplantation of hADSC and hBMSC alone did not show any human engraftment in NOD/SCID mice.

We also determined the level of human CD19 and CD31 for estimating the reconstitution of myeloid and lymphoid lineages. The levels of CD19 and CD31 in the bone marrow were also significantly higher in mice coninfused with hADSC and hBMSC (Table 1).

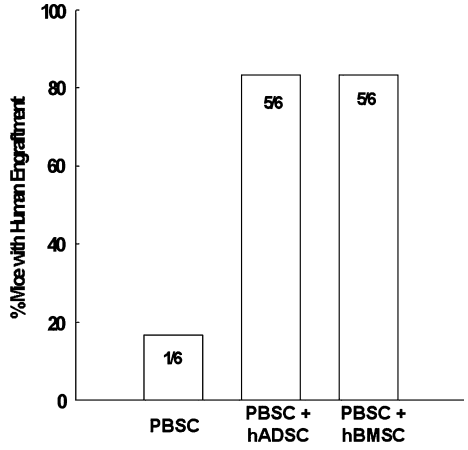

Fig. 4. Frequency of human hematopoietic engraftment in NOD/SCID mice. Following 250 cGy irradiation, NOD/SCID mice were transplanted with 5 × 105 PBSC per mouse with or without 1 × 106 hADSC or hBMSC cultured in HS. Numbers indicate mice with human engraftment/mice transplanted.

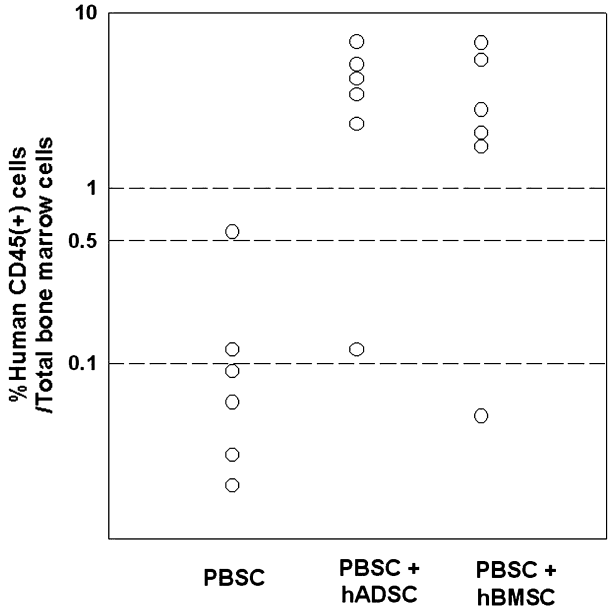

Fig. 5. Percentage human hematopoietic cells (CD45+) in an individual mouse shown in Fig. 4. Bone marrow mononuclear cells were obtained from each mouse and CD45(+) cell percentage was determined by flow cytometry.

Table 1
Effect of contransplantation of hADSC and hBMSC cultured in human serum on engraftment of PBSC in bone marrow and peripheral blood of NOD/SCID mice at 6 weeks after transplantation

	PBSC alone	PBSC + hADSC	PBSC + hBMSC
Bone marrow			
CD45	0.17 ± 0.21	$3.64 \pm 2.30^*$	$3.11 \pm 2.47^*$
CD33	0.05 ± 0.06	$1.12 \pm 0.78^*$	$1.01 \pm 0.61^*$
CD19	0.11 ± 0.09	$2.57 \pm 1.41^*$	$2.22 \pm 1.62^*$
Peripheral blood			
CD45	0.08 ± 0.07	$1.78 \pm 1.05^*$	$1.52 \pm 1.22^*$

Data represent means ± SEM ($n = 6$ per group).
* $p < 0.05$.

Discussion

It has been reported that FBS proteins are internalized into the cells and cannot be removed by simple

Fig. 2. (A) Growth curves of three independent hMSC cultures. Cells were passaged with split ratio 1:10 in culture media containing HS or FBS. Cells were enumerated at each passage under hemocytometer. Growth curve was determined at three different MSC samples and two different lots of human serum were tested for each sample. Data represent means. (B) Comparison of cell morphology cultured in media containing HS or FBS. hADSC (passage 3) were grown in MD or MF containing FBS or HS and photographs were taken under a phase contrast microscope (magnification of 200×).

Fig. 3. Effect of HS on adipogenic and osteogenic differentiation of hMSC. (A) hADSC were cultured at a confluent state before induction of differentiation, and cells were differentiated for subsequent 14 days in adipogenic medium (AM) and osteogenic medium (OM). After then, hADSC were stained with oil red O and alizarin red S. (B) Quantitation of adipogenic and osteogenic differentiation at each culture condition. hBMSC and hADSC were cultured in MF media containing HS or FBS. Differentiation was determined at four different MSC samples and two different lots of human serum were tested for each sample. Data represent means.

washing of cells [30]. Because the internalized FBS can elicit immunogenicity, the development of the method for removal of exposed FBS is critical for clinical trials of MSC in cell and gene therapy. FBS-free culture of MSC will be a safer way in hMSC transplantation into patients. The simple replacement of FBS by HS in α-MEM media did not support proliferation of MSC [32]. In this study, we cultured hMSC without exposure of FBS during the whole isolation steps. hMSC grown in our culture condition can maintain proliferative and differentiation potential for at least 10 passages. Furthermore, MSC cultured in HS can promote engraftment of PBSC in NOD/SCID mice, indicating that the hMSC, which are expanded according to the method described in this study, maintain the characteristics of MSC in vivo as well as in vitro.

In all previous experiments [28,29,40–43] except the study that used human fetal lung-derived MSC [44], MSC derived from bone marrow were used for engraftment experiments of HSC. However, human fetal lung-derived MSC may be more difficult due to the limited availability of such tissues and ethical concerns with procurement. In this study, we first demonstrated that hADSC can be used as an alternative of BMSC for this purpose.

This study showed that allogeneic hADSC as well as hBMSC expanded in HS exhibit the capacity to promote engraftment of PBSC in NOD/SCID mice. Cotransplantation of PBSC and cultured hMSC resulted in significantly higher frequency and levels of human engraftment than was observed after transplantation of PBSC alone. When patients are transplanted with a single unit unrelated PBSC, engraftment failure is approximately 10% and hematopoietic recovery is delayed [40]. This is likely due to the limiting numbers of HS found in a single unit of PBSC compared to conventional allogeneic graft sources. Thus, our data suggest that coinfusion of hADSC in patients undergoing a single donor PBSC transplantation improves engraftment rate and kinetics in hematopoietic stem cell transplantation, especially, of patients whose availability of autologous bone marrow samples is limited.

In conclusion, our data indicate that hMSC cultured in autologous HS can be used without any safety issues raised by FBS contamination in cellular and genetic therapies including promotion of engraftment after allogeneic HSC transplantation.

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