

Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells

Katharina Schallmoser,* Christina Bartmann,* Eva Rohde, Andreas Reinisch, Karl Kashofer, Elke Stadelmeyer, Camilla Drexler, Gerhard Lanzer, Werner Linkesch, and Dirk Strunk

BACKGROUND: Human multipotent mesenchymal stromal cells (MSCs) are promising candidates for a growing spectrum of regenerative and immunomodulatory cellular therapies. Translation of auspicious experimental results into clinical applications has been limited by the dependence of MSC propagation from fetal bovine serum (FBS).

STUDY DESIGN AND METHODS: The capacity of human platelet lysate (HPL) to replace FBS for clinical-scale MSC propagation was analyzed.

RESULTS: HPL could be efficiently produced from buffy coats. Multiplex analyses allowed a distinct HPL growth factor profile to be delineated. With a previously established two-step clinical-scale procedure, HPL was reproducibly more efficient than FBS in supporting MSC outgrowth. With only 3×10^5 primary culture-derived MSCs, a mean of 4.36×10^8 HPL-MSCs (range, 3.01×10^8 - 5.40×10^8) was obtained within a single secondary 11- to 13-day culture step. Although morphologically distinct, HPL-MSCs and FBS-MSCs did not differ significantly in terms of immunophenotype, differentiation potential in vitro, and lack of tumorigenicity in nude mice in vivo.

CONCLUSIONS: Replacing FBS with HPL prevents bovine prion, viral, and zoonose contamination of the stem cell product. This new efficient FBS-free two-step procedure for clinical-scale MSC propagation may represent a major step toward challenging new stem cell therapies.

Multipotent mesenchymal stromal cells (MSCs) are currently undergoing clinical trials to explore their potential regulation of hematopoiesis, immune response, and tissue regeneration (<http://www.clinicaltrials.gov/>). There is already evidence for the safety and measurable efficiency of systemic MSC infusions in various disease conditions.¹⁻¹⁰ MSCs represent less than 0.1% of nucleated cells in adult bone marrow (BM) aspirates. Ex vivo expansion to obtain sufficient amounts of MSCs for a suggested clinical dose of at least 2×10^6 MSCs per kg body weight of the adult patient is a challenge.⁷ With a two-step preclinical protocol we were able to reproducibly generate a minimum of 1.5×10^8 MSCs from 10-mL BM aspirates within 4 weeks in fetal bovine serum (FBS)-supplemented

ABBREVIATIONS: α -MEM = alpha-modified minimum essential medium; bFGF = basic fibroblast growth factor; CFU-F = fibroblast colony-forming unit; EGF = epidermal growth factor; HPL = human platelet lysate; MSC(s) = mesenchymal stromal cell(s); PDGF(s) = platelet-derived growth factor(s); PRP = platelet-rich plasma; VEGF = vascular endothelial cell growth factor.

From the Department of Blood Group Serology and Transfusion Medicine, the Department of Internal Medicine, Division of Hematology and Stem Cell Transplantation, the Institute of Pathology, the Department of Orthopedic Surgery, and StemCell Cluster, Medical University, Graz, Austria.

Address reprint requests to: Dirk Strunk, MD, Department of Internal Medicine, Division of Hematology and Stem Cell Transplantation, Medical University, Auenbrugger Pl. 38, A-8036 Graz, Austria; e-mail: dirk.strunk@klinikum-graz.at.

*Both authors contributed equally.

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cultures.¹¹ The common use of FBS in MSC cultures as a source of growth factors carries the risk of transmission of known and unknown pathogens as well as xenoimmunization against bovine antigens.¹²⁻¹⁷ The ideal situation would be to avoid the use of FBS during human stem cell manipulation.^{18,19} So far optimal MSC propagation has been strictly dependent on FBS-supplemented culture. The reduction of bovine antigens by a final 48-hour incubation with medium supplemented with 20 percent human blood group AB serum to prepare hypoimmunogenic MSCs has been published to improve MSC therapies.²⁰ The obvious risk of the use of animal sera in the process of MSC propagation is the subject of ongoing debate.²¹

Several factors required for MSC growth in vitro have already been identified by antibody-mediated neutralization.^{22,23} Human platelets (PLTs) enriched in PLT-rich plasma (PRP), PLT lysate, or PLT gel contain PLT-derived growth factors (PDGFs), transforming growth factor- β (TGF- β), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF). These growth factors are mitogenic for bone cells, chondrocytes, and MSCs.²⁴⁻³³ Combined application of stromal stem cell and PRP improved bone allograft integration and was associated with increased vascular invasion and improved bone repair.³⁴ Topical application of PLT derivatives to support tissue regeneration and wound healing has been studied under heterogeneous and nonstandardized conditions.^{29,35-37} The observed regenerative effects of PLT derivatives and their clinical outcome have varied widely.^{24,38-40} In vitro studies have indicated that the content of growth factors varies according to PLT concentration, preparation mode, white blood cell (WBC) contamination, and mechanism of PLT growth factor release.⁴¹⁻⁴⁴ Duration of exposure and local PLT concentration can also influence endothelial cell, fibroblast, and osteoblast function in vitro.^{27,28,45}

Most recently Doucet and coworkers³² have shown that MSCs cultured in the presence of PLT lysate under laboratory-scale conditions with a seeding density of 1000 or 6000 per cm² maintained their differentiation properties and immunosuppressive activity. We previously developed a reliable two-step procedure for the clinical-scale propagation of functional human MSC in an FBS-dependent system.¹¹ Taking into consideration the variable effects of human PLT-derived factors in different safe clinical applications, we succeeded in fully replacing FBS with human PLT lysate (HPL) as the key culture medium substitute for clinical-scale MSC production.

MATERIALS AND METHODS

Preparation of HPL

Whole blood (450 ± 45 mL) was collected with informed consent from random donors. Collection followed the

Austrian regulatory procedures for blood donation into a standard citrate phosphate dextrose top-and-bottom quadruple bag (MacoPharma, Tourcoing, France). In accordance with the Austrian and European guidelines, routine testing of blood donors was performed for the following: ABO blood groups, irregular red blood cell (RBC) antibodies, and infections (by polymerase chain reaction [PCR] testing for hepatitis C virus, hepatitis B virus, human immunodeficiency virus-1 and -2, hepatitis A virus, and parvovirus B19 and by treponema pallidum hemagglutination assay testing for *Treponema pallidum*). Furthermore, analysis of peripheral blood cell count was performed and donors with WBC counts above 12×10^3 per μ L were excluded. After a resting period of 16 hours at $22 \pm 2^\circ\text{C}$, the blood units were centrifuged ($4250 \times g$, 13 min, 22°C). RBCs and plasma were separated automatically (Compomat G3, NPBI, Amsterdam, the Netherlands) from the buffy-coat fraction and transferred into satellite containers. Four buffy-coat units of blood group O and 1 unit of plasma blood group AB were pooled to avoid exposure of MSC to ABO blood group antigens and isoagglutinins and were centrifuged with soft centrifugation ($341 \times g$, 6 min, 22°C) to comprise 1 unit of PRP. The PRP was transferred into a storage bag after inline filtration for WBC depletion (PALL Autostop, Pall, East Hill, NY) and the PRP was frozen at -30°C . Regular quality control of PRP included PLT concentration and WBC and RBC contamination. Microbiologic testing of aliquots for sterility was performed with a bacterial detection system (BacT/ALERT, bioMérieux, Marcy-Etoile, France).⁴⁶ After thawing at 37°C for PLT fragmentation, a minimum of 10 units of lysed human PRP (representing 40 buffy coats and 10 plasma units) was pooled to avoid individual donor variations. The resulting HPL pool was stored at -30°C until use. HPL was centrifuged after thawing ($4000 \times g/15$ min) to reduce the presence of PLT membrane fragments as a potential risk for alloimmunization against MHC Class I and human PLT antigens. The supernatant was added to heparinized alpha-modified minimum essential medium (α -MEM) before filtration through a 0.22- μ m sterile bottle filter (SCGPU05RE, Millipore, Billerica, MA).

Collection of BM cells

A maximum of 10 mL of BM aspirate in 2.5-mL aliquots was obtained from healthy donors ($n = 4$; age, 9-43 years; female:male ratio, 2:2) in 5-mL syringes loaded with 500 IU of preservative-free heparin (Biochrom AG, Berlin, Germany) with informed consent.¹¹

Primary MSC culture (Step 1)

BM was diluted immediately after harvest in α -MEM (M4526, Sigma-Aldrich, St Louis, MO) supplemented with 2 mmol per L L-glutamine (Sigma), 25 mmol per L HEPES

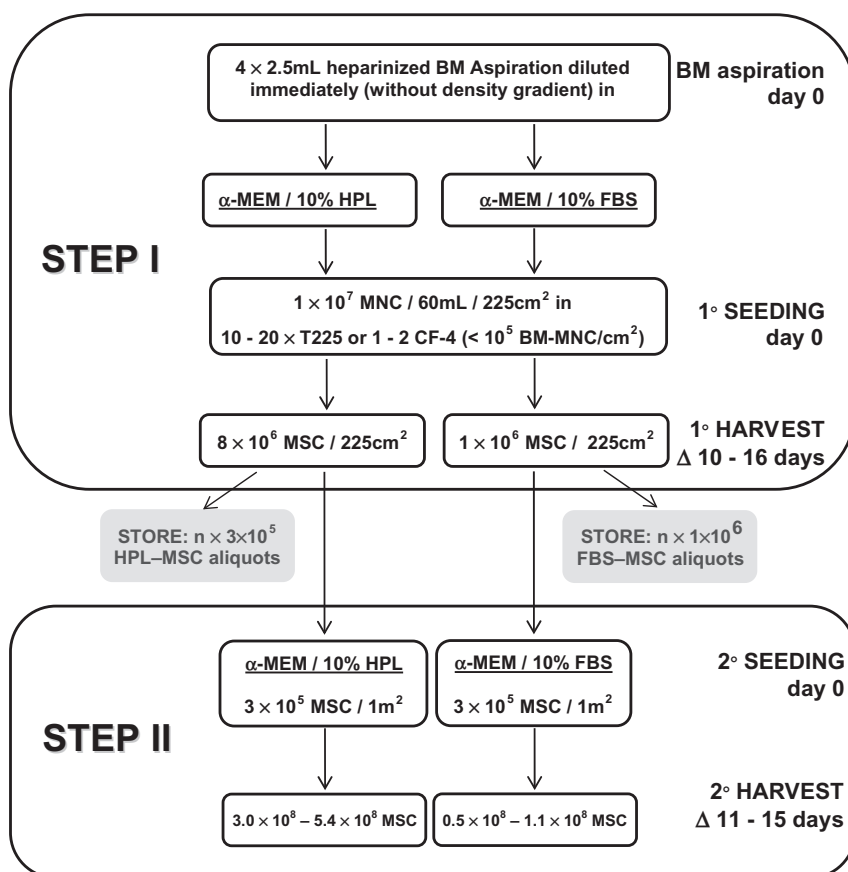


Fig. 1. Executive summary of the two-step MSC production. Seeding and harvest numbers of BM-MNCs and resulting MSCs in HPL-supplemented compared to FBS-supplemented α -MEM as well as storage of aliquots of harvested MSC are indicated to summarize the two steps to clinical MSC quantity.

(Sigma), 100 U per mL penicillin, and 100 μ g per mL streptomycin (Gibco Cell Culture, Invitrogen Corp., Grand Island, NY). For direct comparison, each BM sample was expanded in α -MEM additionally supplemented with either 10 percent FBS (HyClone, Logan, UT) or 10 percent HPL. Preservative-free heparin (2 U/mL; Biochrom AG) was added before HPL supplementation to the medium to avoid PLT gel formation. No effect on MSC proliferation was observed after addition of heparin (2 U/mL) to FBS-supplemented cultures in pilot experiments (data not shown). BM mononuclear cell (MNC) content was analyzed by automated blood count (Coulter Onyx, Beckman Coulter, Fullerton, CA). Cells were seeded at a density corresponding to 0.57×10^4 to 16×10^4 MNCs per cm^2 in 3 to 10 tissue culture flasks ($1.32 \times 10^7 \pm 1.14 \times 10^7$ MNCs/ 225 cm^2 ; Corning Inc., Acton, MA) and cultured at 5 percent CO_2 , 37°C , 95 percent air humidity. Nonadherent cells were removed by complete change of medium after 2 to 3 days. The cultures were fed twice weekly by replacing 30 percent of the medium with fresh supplemented medium. Cells were harvested before reaching

confluence (at Days 10-16) by application of 0.25 percent trypsin 1 mmol per L ethylenediaminetetraacetate (1-5 min, 37°C ; Gibco). Nucleated cell numbers were determined from the mean of four measurements with a hemocytometer. Documentation of cell cultures and determination of fibroblast colony-forming units (CFU-F) were performed with previously published procedures.^{11,47} MSC aliquots were frozen after primary culture, kept in liquid nitrogen, and rethawed for experimental and preclinical expansion experiments as indicated (Fig. 1).

Large-scale MSC expansion (Step 2)

For large-scale expansion aliquots of 3×10^5 MSCs derived from primary culture were seeded in α -MEM/10 percent HPL and α -MEM/10 percent FBS with a seeding density of 30 per cm^2 on 1 m^2 culture area in four four-layered cell factories (CF-4, Nalge Nunc International, Naperville, IL) and cultured to reach confluence. Two of the four expansions were performed with rethawed samples frozen after primary culture. Medium change (30%) was performed twice weekly, and MSCs were harvested on Days 11 through 15 by trypsinization (Fig. 1).

Condensed two-step clinical-scale MSC production procedure

For clinical-scale MSC production, BM aspiration was performed in a sterile environment. Primary BM culture (Step 1 of the clinical-scale MSC production) and consecutive large-scale expansion (Step 2 of the clinical-scale MSC production) were performed in a good manufacturing practice laboratory by specially trained personnel. Nucleated cell numbers were determined from the mean of four measurements with a hemocytometer. Viability was ascertained by trypan blue exclusion. On days of MSC seeding and harvest, sterility testing of aliquots of medium supernatant was performed to exclude fungal and bacterial contamination with the BacT/ALERT system (bioMérieux).⁴⁶ Mycoplasma PCR was conducted on days of harvest by following the manufacturer's instructions (VenorGeM, Minerva Biolabs, Berlin, Germany). Cryopreservation was performed as described.¹¹

Long-term expansion

To evaluate the long-term growth kinetics in FBS- and HPL-supplemented media, an aliquot of MSCs harvested after clinical-scale expansion (i.e., after primary culture plus one passage of large-scale expansion; $n = 4$) was further propagated for three additional passages. These cultures were performed at the same density as the large-scale expansion ($30/\text{cm}^2$) in the same media (HPL-supplement vs. FBS-supplemented α -MEM) in two to four 225- cm^2 tissue culture flasks per group (Corning Inc.). The estimated total MSC yield and cumulative population doublings were calculated for primary culture plus four passages (approx. 8 weeks).

Multiplex growth factor analysis

Aliquots of cell culture media supplemented with FBS or HPL before use on Day 0 and cell culture supernatants on the day of MSC harvest were frozen at -70°C . Multiplex human growth factor detection (Beadlyte human growth factor beadmates, Upstate Biotechnology, Lake Placid, NY) was performed with a chemiluminescence detector according to the manufacturer's instructions (Luminex Corp., Austin, TX). This was used to measure the concentration of EGF, bFGF, PDGF-AA, PDGF-AB/BB, and vascular endothelial cell growth factor (VEGF; detection limit, 14 pg/mL for VEGF, 7 pg/mL for all other growth factors).

Differentiation assays

Osteogenic differentiation assays were performed with MSC derived from FBS- and HPL-supplemented cultures from two donors after clinical-scale expansion, and assays were visualized with alizarin red staining.⁴⁸ Adipogenic differentiation of the same two MSC samples was performed as described except that ascorbic acid was replaced by insulin.⁴⁹

Multiparameter flow cytometry

The surface marker expression of MSC expanded in FBS- and HPL-supplemented media were analyzed with a four-color flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) as described previously.¹¹ Briefly, MSCs were washed and blocked with sheep serum. They were then stained for 25 minutes at 4°C with fluorochrome-labeled BS-1 lectin and monoclonal antibodies against HLA-AB (Harlan Sera-Lab, Leicestershire, UK), HLA-DR, CD5, CD10, CD13, CD14, CD29, CD31, CD34, CD45, CD56, CD73, CD90 (all Becton Dickinson PharMingen), CD105 (Caltag Laboratories, Burlingame, CA), and CD146 (clone P1H12, Chemicon International, Temecula, CA). Appropriate isotype-matched antibodies were used as negative controls (Becton Dickinson). Four-color mea-

surements were performed, and data from a minimum of 10,000 viable propidium iodine-excluding cells were stored. List mode files were analyzed with computer software (CellQuest Pro and Paint-A-Gate Pro, Becton Dickinson).

In vivo tumorigenicity test

Animal experiments were performed in accordance with institutional guidelines. To test for tumor formation potential of ex vivo-propagated MSCs in immunocompromised hosts, athymic nude mice (Harlan, Indianapolis, IN) were injected subcutaneously with 2×10^6 or 2×10^4 MSCs derived from clinical-scale expansions in α -MEM/HPL and α -MEM/FBS (four groups with three animals per group). Mice were observed for 90 days before euthanasia and excision of the marked MSC injection site. Controls were two groups of two additional nude mice each inoculated subcutaneously on Day 88 with 2×10^6 (right flank) and 2×10^4 MSCs (left flank) derived from the same clinical-scale expansions in α -MEM/HPL and α -MEM/FBS. These were also euthanized on Day 90 to determine cell retrieval. Histologic analyses were performed as described.⁵⁰

Statistical analysis

Computer software (SPSS 11.5.1, SPSS Inc., Chicago, IL) was employed for statistical analyses. Statistical differences were assessed with a *t* test. Significance was set at a *p* value of less than 0.05.

RESULTS

Characteristics of HPL

We were able to produce batches of 3.0 to 3.5 L of pooled PRP representing material from at least 50 individual donors. The PLT concentration in PRP was $0.95 \times 10^9 \pm 0.15 \times 10^9$ per mL (mean $\pm$ SD). Routine testing of the blood donors was negative for irregular RBC antibodies and infection markers. Remaining WBC contamination after inline filtration for WBC depletion was continually below a threshold of 1×10^6 per unit ($0.07 \times 10^6 \pm 0.09 \times 10^6$ /unit; mean $\pm$ SD). RBC contamination was $0.51 \times 10^9 \pm 0.29 \times 10^9$ per unit. Significant amounts of EGF and PDGFs with less than 6 percent variation and bFGF with 14 percent variation between the three batches tested could be measured after supplementing α -MEM medium with 10 percent HPL immediately before culture initiation (Table 1). Tests for sterility (bacterial or fungal contamination) before and after culture as well as mycoplasma PCR were continually negative.

Clinical-scale expansions

In the first step, the expansion procedure was initiated from $1.32 \times 10^7 \pm 1.14 \times 10^7$ MNCs per 225 cm^2 . MSCs

TABLE 1. Mean growth factor concentrations in medium supernatants*

Growth factor	EGF		bFGF		PDGF-AA		PDGF-AB/BB		VEGF	
	Day 0	Day 13	Day 0	Day 13	Day 0	Day 13	Day 0	Day 13	Day 0	Day 13
FBS	0	9 ± 6	25 ± 3	41 ± 6	0	139 ± 5	0	87 ± 15	0	746 ± 22
HPL	192 ± 11	56 ± 13	64 ± 9	50 ± 5	7,424 ± 409	2,017 ± 1,043	40,458 ± 1,791	4,655 ± 1,353	0	2,481 ± 136†

* Growth factor concentration in α -MEM supplemented with 10 percent FBS and 10 percent HPL on Day 0 and cell culture supernatants on Day 13 of clinical-scale expansion of MSCs measured by multiplex growth factor analysis (pg/mL; n = 3; mean $\pm$ SEM).

† p = 0.00003.

were successfully grown to $8.02 \times 10^6 \pm 1.22 \times 10^6$ with α -MEM/10 percent HPL and to $1.46 \times 10^6 \pm 1.05 \times 10^6$ with α -MEM/10 percent FBS per 225-cm² flask within 10 to 16 days of primary culture. This revealed a significantly higher proliferation of MSC stimulated by human PLT-derived factors (p = 0.009). Within the first culture period HPL-driven cultures showed a delayed MSC outgrowth until Day 6. This delay was reproducibly compensated by an advanced late proliferation enhancement resulting in continually higher MSC harvests at the end of the primary culture with HPL compared to FBS (Fig. 2A). Microscopic examination revealed a smaller cell size and more elongated morphology (Fig. 2B). CFU-F analyses further showed increased colony size and density due to an increased MSC number within single HPL-stimulated colonies but no significant differences in colony number (Fig. 2C and data not shown).

Step 2 of the clinical-scale expansion was initiated with only 3×10^5 primary culture-derived MSC on a growth area of 1 m² (in four CF-4). This protocol was based on preliminary experience derived from experimental laboratory-scale MSC cultures with α -MEM/10 percent HPL (data not shown). After 11 to 15 days of secondary culture, we obtained $4.36 \times 10^8 \pm 0.51 \times 10^8$ HPL-MSCs compared to $7.49 \times 10^7 \pm 1.62 \times 10^7$ FBS-MSCs (mean $\pm$ SEM) representing 10.49 compared to 7.98 population doublings, respectively (Fig. 3A). Whereas the above-described morphologic differences between cells in HPL- and FBS-driven cultures remained during large-scale expansion, this did not translate into significant immunophenotypic differences. Flow cytometry revealed that MSCs were positive for the presence of HLA-AB, CD13, CD29, CD73, CD90, CD105, CD146, and BS-1 and negative for the presence of HLA-DR, CD5, CD10, CD14, CD31, CD34, CD45, and CD56 regardless of whether the MSCs had been generated with HPL or FBS (Fig. 4 and Table 2).

MSC functionality and lack of tumorigenicity

Growth factor profiling at the start and the end of the large-scale expansion showed consumption of PDGFs during MSC culture and a significantly higher VEGF production by MSCs in HPL compared to FBS cultures (p = 0.00003; Table 1). In vitro assays after clinical-scale

expansions revealed intact osteogenic and adipogenic differentiation potential (Fig. 5).

Aliquots of all four BM samples derived from the clinical-scale expansions after Step 2 were further subjected to long-term expansion. This resulted in a calculated final cell product of a mean of 6.31×10^{15} MSCs in HPL-driven cultures and a mean of 8.65×10^{14} MSCs in FBS-driven cultures (range, 3.45×10^9 - 2.52×10^{16} and 2.10×10^{12} - 2.45×10^{15} , respectively). We found a cumulative mean of 23.97 and 23.44 population doublings with HPL and FBS, respectively (Figs. 3B and 3C). Notably the significant initial growth advantage in HPL (p = 0.012) was overtaken by a significant increase of population doublings in FBS-containing cultures between Day 51 and Day 68 (p = 0.028; Fig. 3C). So far, we have not observed outgrowth of immortal cell lines from extended BM-MSC cultures. Because conventional cytogenetic testing did not reliably produce sufficient numbers (i.e., ≥ 20) of metaphases in two-thirds of our samples, we tested for tumorigenicity by injecting MSC into athymic nude mice. Subcutaneous injection of 2×10^6 and 2×10^4 MSCs derived from clinical-scale expansions under both HPL and FBS stimulation produced a visible primary cell deposit. After 48 hours, injected MSCs could be recovered and visualized by conventional microscopy (Fig. 6). None of the 12 animals tested developed a visible or microscopically detectable tumor over a 90-day observation period. Microscopic analysis of serial sections indicated that the injected human MSCs had completely disappeared.

DISCUSSION

We have demonstrated that HPL can fully replace FBS as a culture substitute for clinical-scale human MSC expansion. In four of four cases, more than 2×10^6 functional MSCs per kg could be generated for a 75-kg adult with a practicable two-step expansion procedure. The efficiency of MSC expansion with HPL compared to FBS resulted in a reduction of the cell culture area necessary during the second (large-scale) expansion. Furthermore, the FBS-free MSC propagation prevents bovine pathogen transfer and xenoimmunization. HPL-driven MSC propagation thus bears the potential to facilitate rational cell dose-escalated MSC therapies in adult patients which to date have been partly hampered by limited MSC quantities.^{2,7} It

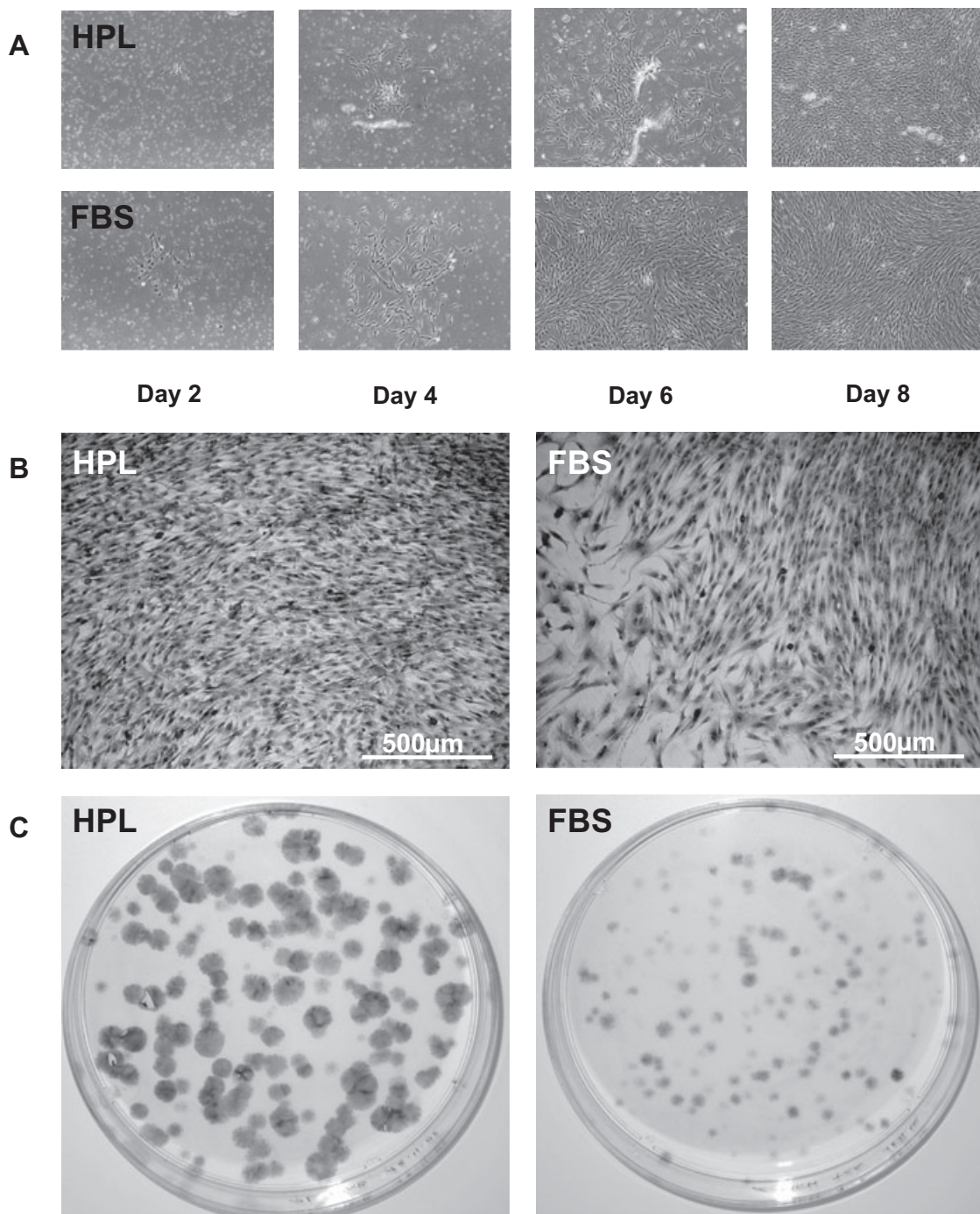
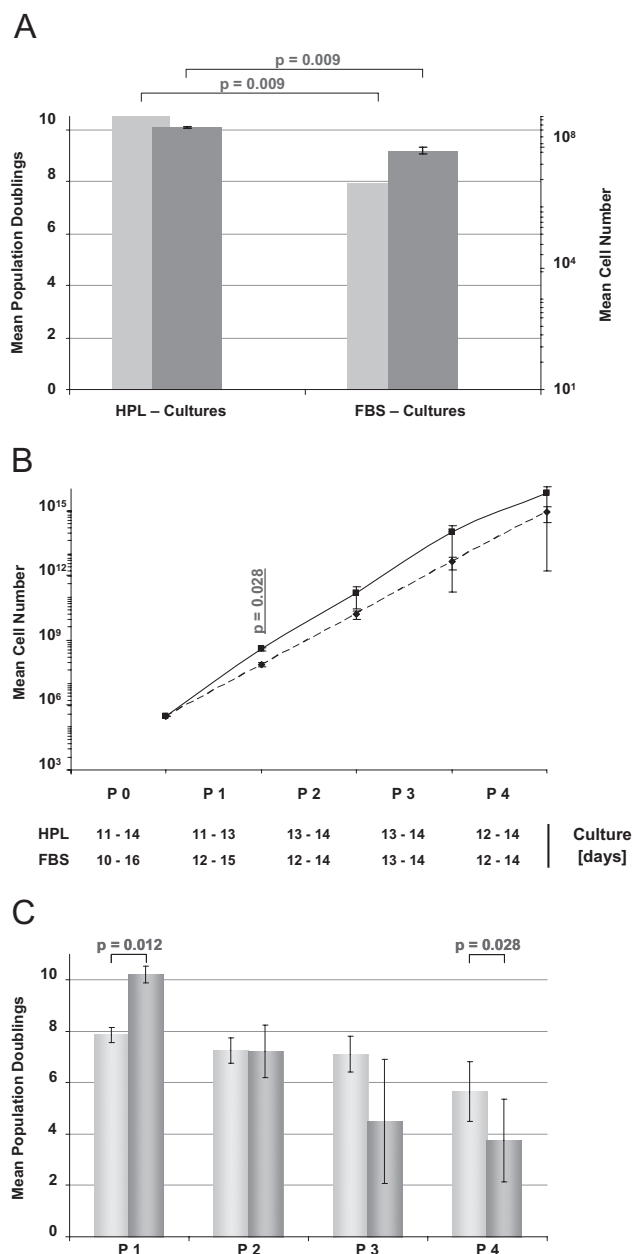


Fig. 2. MSCs in primary culture. (A) Two examples of plastic adherent colonies of cells derived from the same heparinized primary human BM aspiration directly diluted (without density gradient centrifugation) to a density corresponding to 1×10^4 MNCs per mL in α -MEM supplemented with either 10 percent HPL (top row) or FBS (bottom row). Nonadherent cells visible on Day 2 have been removed by replacing the medium on Day 3 as described under Materials and Methods. Colony growth has been documented every second day as indicated (40 $\times$ magnification). (B) Primary culture MSCs propagated in HPL- and FBS-supplemented medium show differences in morphology and (C) CFU-F density (Day 12; 40 $\times$ magnification).



is not clear whether the more robust MSC cultures produced by HPL only resulted from the rather constant EGF, FGF, and PDGF activities measured in our pooled HPL preparation. Further studies are needed to better understand the behavior of MSC prepared with HPL compared to that with FBS.

For economic reasons, long-term expansions in this study were only performed with aliquots of MSCs derived from the second step of the clinical-scale MSC production. A cumulative growth capacity of a mean of 6×10^{15} total MSCs in HPL and 8×10^{14} in FBS-supplemented cultures after primary culture and only four additional passages (corresponding to a maximum of 68 culture days) was calculated. The fact that MSCs eventually stopped

Fig. 3. Clinical-scale and long-term expansions. (A) Clinical-scale expansions were performed in a good manufacturing practice laboratory on 1-m² culture area in four 4-layered cell factories. MSCs derived from 11- to 16-day primary BM cultures were seeded in α -MEM/HPL (left) and α -MEM/FBS (right) at 30 per cm² (3×10^5 /1 m²). Mean population doublings (light gray) and cell number (dark gray) after 11 to 15 days of secondary culture result from harvest of four different donors. (B) After each clinical expansion (P1) an aliquot of MSC was further propagated at less than 50 per cm² in HPL- (solid line) and FBS-driven cultures (dashed line). Calculated total MSC yield and cumulative population doublings for three additional passages (until approx. Day 65) are shown. P0 indicates primary cultures of unseparated BM aspirates (diluted in the respective medium) with the primary MSC number being represented by the curve gap. Each cycle of reseedling of MSCs after trypsinization was considered as one additional passage with P1 starting from standardized 3×10^5 P0-derived MSCs. (C) Population doublings of MSC cultured in FBS- (light gray) and HPL-supplemented media (dark gray) were calculated after each passage of long-term expansion ($n = 4$; mean $\pm$ SEM).

growing after more than 10 weeks of culture at least in part argues against an inherent transformation potential. The observation that these human MSC did not form tumors in nude mice may further argue against tumorigenicity in vivo. Whether the disappearance of the MSC in the experimental animals results from mere cell death or rejection by remaining NK or other immune cell activities in the nude animals remains to be determined.

Although the risk of transferring putative pathogens and xenogeneic material is reduced by replacing FBS, further investigation is required to substantiate the equivalence of MSCs propagated with HPL. Retained immunosuppressive activity of FBS-free laboratory-scale expanded MSCs has already been demonstrated.³² The observation that they maintain their differentiation properties has also been made by Doucet and colleagues³² and has been confirmed in this study after clinical-scale expansion. A series of reverse transcription-quantitative PCR experiments is currently being planned to address the question of possible quantitative differences in the differentiation capacity. So far we can only speculate that the human growth factors detected in HPL are mediating some of the observed differences. Questions unresolved during this early phase of work with HPL, however, relate to "simple" differences in proliferation, cell size, and CFU-F morphology through to complex biologic process regulations. After decades of work with FBS as a rather artificial growth stimulus for human cells, it will be fascinating to learn how human growth factors released from PLTs regulate MSC behavior. Blocking experiments and functional interference downstream from the growth

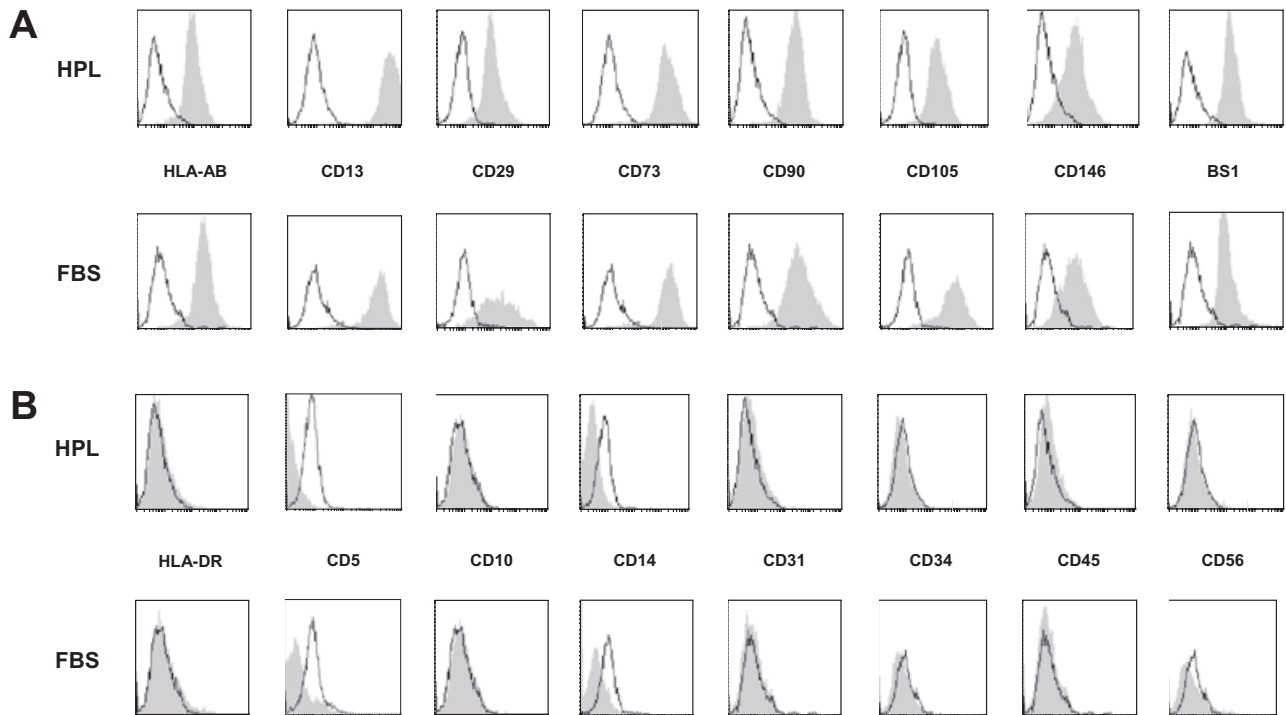


Fig. 4. MSC immunophenotype. Flow cytometry analysis revealed that large-scale expanded MSCs in HPL- and FBS-driven cultures consistently stained positive for (A) HLA-AB, CD13, CD29, CD73, CD90, CD105, CD146, and BS-1 lectin and did not react with (B) HLA-DR, CD5, CD10, CD14, CD31, CD34, CD45, and CD56 ($n = 4$; one representative example shown).

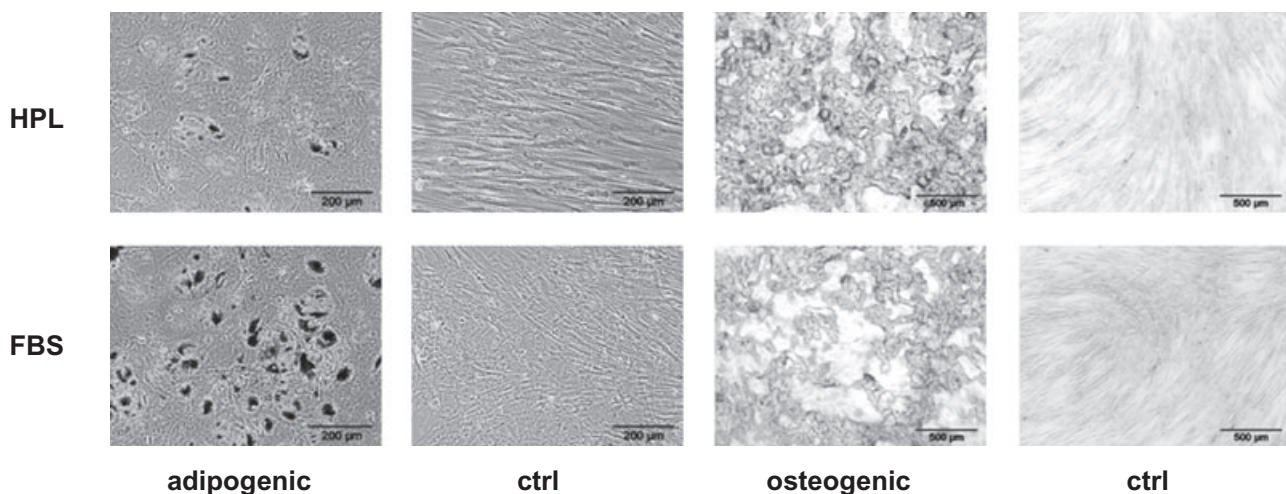


Fig. 5. Osteogenic and adipogenic differentiation. Clinical-scale expanded MSCs from HPL- and FBS-driven cultures were in vitro differentiated into adipogenic and osteogenic cell lineage ($n = 3$; one representative example shown).

factor receptors will be one way to understand mechanisms of regulation. To gain broader insight into the differences under HPL compared to FBS stimulation, a complete transcriptome analysis of human MSCs recovered from the clinical-scale expansions has already been executed. This is currently extended by mRNA quantification and additional functional testing (C. Bartmann et al, manuscript in preparation). Provided that cumulative

results signify continuous efficiency and safety of HPL-expanded MSCs, this would represent a major step toward novel stem cell therapies. Further investigation will also help to understand the peculiar role that PLT-derived factors may play under various physiopathologic conditions. It can be speculated that the production of VEGF by MSCs during HPL stimulation may be translated into new vascular regenerative concepts.

TABLE 2. Mean fluorescence intensity (MFI) measured with flow cytometry*

	HLA-AB	CD13	CD29	CD73	CD90	CD105	CD146	BS-1
FBS	148.3 ± 28.6	1765.7 ± 207.5	82.5 ± 16.1	908.4 ± 182.7	379.9 ± 224.7	170.3 ± 37.4	94.9 ± 51.0	162.1 ± 29.6
HPL	155.5 ± 29.1	2264.9 ± 328.7	73.0 ± 10.4	806.5 ± 140.1	319.4 ± 151.7	150.0 ± 25.6	70.4 ± 21.8	208.5 ± 51.4

* MFI of positively stained surface markers of MSCs expanded in FBS- and HPL-supplemented cultures (n = 4, mean ± SEM).

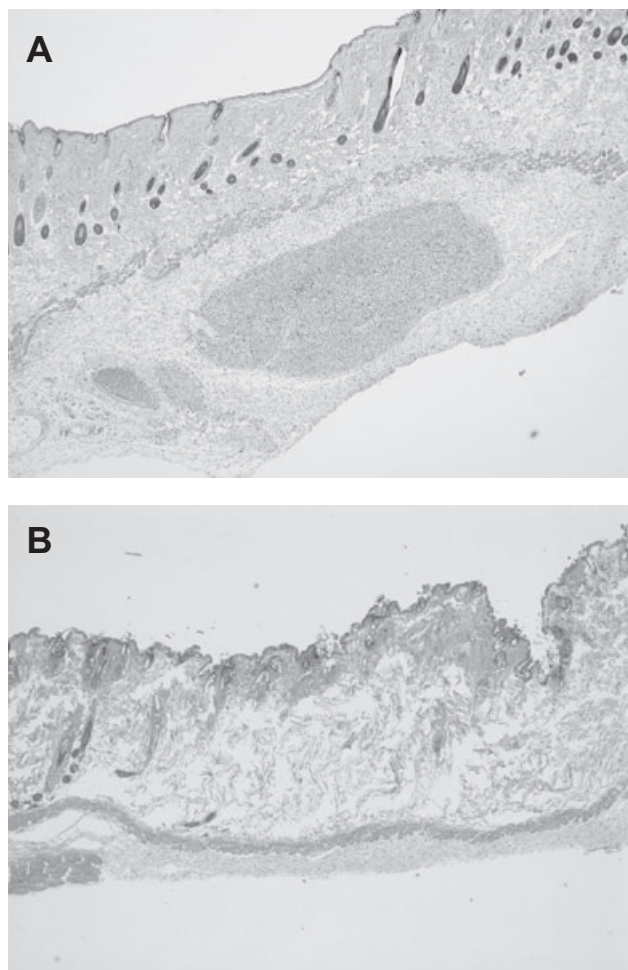


Fig. 6. In vivo tumorigenicity test. Representative H&E stain of athymic nude mouse skin sections (A) 48 hours and (B) 90 days after inoculation of 2×10^6 human MSCs derived from large-scale expansion in HPL-supplemented α -MEM (details given under Materials and Methods and Results).

Based on the accumulating evidence showing the efficiency and safety of HPL or equivalent human PRP-derived formulations, the discussion of the “best” way to propagate human MSCs for different therapeutic purposes has now been reopened. Additional studies are vital. The efficient clinical-scale propagation of human MSCs with HPL as described in this study not only eliminates the risk of bovine pathogen contamination and xenoimmunization. It may also support attempts of dose escalation to define the effective MSC dose for different indications.

Ideally it can help to pave the way toward better standardized and safe MSC products with the advantage of improved comparability between prospective randomized clinical trials.

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