Xeno-Free Cultivation of Mesenchymal Stem Cells From the Corneal Stroma

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Purpose. The human cornea has recently been described as a source of corneal stroma–derived mesenchymal stem cells (hMSCs). In vitro expansion of these cells involves basal medium supplemented with fetal bovine serum (FBS). As animal-derived serum can confer a risk of disease transmission and can be subject to considerable lot-to-lot variability, it does not comply with newer Good Manufacturing Practice (GMP)-required animal component–free culture protocols for clinical translation.

Methods. This study investigated animal-free alternatives to FBS for cultivation of human corneal stromal MSCs. Proliferative capacity was studied for cultures supplemented with different concentrations (2.5%, 5%, and 10%) of FBS, human AB serum, human platelet lysate (HPL), and XerumFree. Unsupplemented basal medium was used as a control. The expression of specific hMSC markers ($CD73^+$, $CD90^+$, $CD105^+$, $CD19^-$, $CD34^-$, $CD79\alpha^-$, $CD11b^-$, $CD14^-$, $CD45^-$, and HLA-DR^-) and trilineage differentiation (adipogenesis, osteogenesis, and chondrogenesis) were compared for the two outperforming supplements: 10% FBS and HPL.

Results. HPL is the only consistent non-xeno supplement where hMSC cultures show significantly higher proliferation than the 10% FBS-supplemented cultures. Both FBS- and HPL-supplemented hMSC cultures showed plastic adherence and trilineage differentiation, and no significant differences were found in the expression of the hMSC marker panel. No significant differences in stemness were detected between FBS and HPL cultures.

Conclusions. We conclude that HPL is the best supplement for expansion of human corneal stromal MSCs. HPL significantly outperforms human AB serum, the chemically defined XerumFree, and even the gold standard, FBS. The xeno-free nature of HPL additionally confers preferred standing for use in GMP-regulated clinical trials using human corneal stromal MSCs.

Keywords: corneal MSCs, xeno-free cultivation, human platelet lysate, fetal bovine serum

Various populations of viable adult mesenchymal stem cells (hMSCs) have been described in the human body.[1] The first viable adult mesenchymal stem cells were isolated from the bone marrow and spleen of guinea pigs, characterized and described by Friedenstein et al.[2,3] in 1970. This has led to numerous research groups exploring the potential of the field. In 1991 Caplan[4] introduced the term mesenchymal stem cells as a collective term for nonhematopoietic stem cell populations. The International Society for Cellular Therapy (ISCT) has since defined minimal criteria that must be fulfilled in order to characterize a certain population of cells as MSCs.[5] The first criterion is plastic adherence, followed by trilineage differentiation potential (osteogenesis, adipogenesis, and chondrogenesis), and finally, the positive expression of stem cell markers CD73, CD90, and CD105 but not CD19, CD34, CD79α, CD11b, CD14, CD45, and HLA-DR.

Currently a total of 656 clinical trials using MSC therapies are known to be in progress or have been completed (accessed January 2017; Table 1; source clinicaltrials.gov). Asia, specifically China, is the leading region based on the number of ongoing clinical trials, followed by Europe and the United States. The most commonly used MSC sources for these cellular therapies are the umbilical cord, peripheral blood, bone marrow, and adipose tissue,[1] but MSCs can be found in most tissues of the body. These smaller MSC sources may also display applications in regenerative medicine. One of the recently discovered smaller MSC sources is the stroma of the human cornea. Branch et al.[6] have characterized corneal stromal cells and identified them in 2012 as being a source of MSCs according to the ISCT criteria. This opened the doors to research in ophthalmology focusing on the use of corneal-derived MSCs in ophthalmologic clinical applications.

There is a severe global shortage in human donor corneas, and as a result there have been considerable efforts to regenerate the corneal stroma in vitro. Corneal MSCs could play an important role in the development of wound healing therapies following severe ocular trauma where standard corneal transplantation may not be possible.[7–9] The ease of cultivation of MSCs, together with their lack of human leukocyte antigen (HLA) expression, makes them the ideal candidate from an allogeneic therapeutic point of view.[10] In order for corneal mesenchymal stem cell therapy to be used clinically, however, the protocols for production of these cellular therapeutics should comply with Good Manufacturing

Table 1. Distribution of Currently Ongoing MSC Therapy Clinical Trials per Geographic Region

Region	Distribution
Asia	249
Europe	137
United States	123
Middle East	63
South America	18
Africa	14
Canada	11
Central America	10
Oceania	8
Other	23
Global total	656

Practice (GMP) regulations.[11] Like most MSC expansion protocols, cultivation of corneal MSCs involves a basal medium, most often Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal bovine serum (FBS). Serum supplementation provides the cells with additional nutrients and growth factors; however, there are drawbacks to the use of animal-derived serum. Although considered the gold standard in MSC expansion protocols, FBS entails a number of drawbacks that have mostly been disregarded because of the lack of suitable alternatives. The market for FBS is inadequately regulated, leading to abuse in both the past and the present.[12–16] Qualitative and quantitative differences due to geographic and seasonal influences result in lot-to-lot variability. FBS is generally ill defined, and immunologic reactions against xenogeneic antigens cannot be excluded. It has been shown that FBS proteins associate with major histocompatibility complex (MHC) class I in long-term cultures, leading to T-cell proliferation even in an autologous setting.[17] Pathogens such as mycoplasma, endotoxin, viruses, and prions cannot be excluded completely. Lastly, concerns have been raised about the mismatch between the global demands and supplies of FBS and from an ethical, animal welfare point of view since FBS is harvested from bovine fetuses.[12,18] This has led to GMP regulators demanding xeno-free culture protocols.[19] In the first study of its kind, we investigate the use of commercially available supplements for human corneal MSC cultivation.

Human platelet lysate (HPL) has gained popularity since first used for MSC expansion in 2005.[20] HPL is obtained through repeated freeze and thaw cycles to disrupt the plasma membrane of human blood platelets, thereby releasing cytokines and other growth factors such as transforming growth factor-β, insulin-like growth factor 1, platelet-derived growth factor, platelet factor 4, basic fibroblast growth factor, and epidermal growth factor.[18,21–23] Even though the growth factors are undoubtedly important for hMSC expansion, the composition of HPL has not yet been fully defined. Nonetheless, HPL has been proven to be a valuable supplement in enhancing MSC proliferation, and it has been argued that platelet units are more standardized than FBS.[18] Here we investigated if HPL can serve as a replacement for FBS and other non–animal-derived supplements such as human AB serum and the chemically defined XerumFree (XF).

Materials and Methods

Media and Supplements

Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies Corporation, Grand Island, NY, USA) was supplemented with 1 μg/mL amphotericin β (Gibco, Life Technologies Corporation) and 10 μg/mL gentamicin (Gibco, Life Technologies Corporation) and used as basal medium in all experiments. The culture medium was completed with either fetal bovine serum (FBS) (Life Technologies Corporation), XerumFree (XF) (TNC Bio BV, Eindhoven, The Netherlands), human AB serum (HAB) (Life Technologies Corporation), or stemulate human platelet lysate (HPL) (Cook Medical, Bloomington, IN, USA).

Table 2. Conditions at Which Cells Were Seeded in the Comparative Experiment Testing 13 Conditions Simultaneously

Basal Medium	Supplement Added	Donors	Replicates	Total No. of Cultures/ Condition
DMEM	10% FBS	3	3	9
DMEM	5% FBS	3	3	9
DMEM	2.5% FBS	2	2	4
DMEM	10% HPL	3	3	9
DMEM	5% HPL	3	3	9
DMEM	2.5% HPL	2	2	4
DMEM	10% HAB	3	3	9
DMEM	5% HAB	3	3	9
DMEM	2.5% HAB	2	2	4
DMEM	10% XF	3	3	9
DMEM	5% XF	3	3	9
DMEM	2.5% XF	3	1	3
DMEM	No supplement	3	3	9

Isolation of Corneal Stromal hMSCs

This study was approved by the local Antwerp University Hospital (UZA), Edegem, Belgium ethics committee (EC number: 14/30/319), and only donor corneas with no objection to donation but unsuitable for corneal banking were used in this study. The enucleated eyes were decontaminated by a series of 1-minute washes in 50% povidone-iodine and 3 × 5 minutes in phosphate-buffered saline (PBS). The corneas were then isolated using a 16-mm trephine. The epithelium was removed through a dispase digestion (Dispase II; Roche, Sigma-Aldrich, Vilvoorde, Belgium—protocol by Espana et al. 2003[24]) followed by a manual peeling of the Descemet's membrane with the endothelial cells. The remaining corneal stroma was sectioned and placed in a tube containing 2 mL of 1 mg/mL collagenase (Sigma Aldrich, Diegem, Belgium) for 4 hours with continuous gentle rocking at 37°C followed by a centrifugation wash step at 200*g* for 5 minutes. Cells were then seeded in basal medium with the supplement of choice for use in the following experimental setups. Cells were passaged at 80% to 90% confluence using TrypLE (Life Technologies Corporation), and in all following experiments early passage (P) cells were used (P4 or lower). The first set of experiments investigated cell proliferation, and the two best-performing conditions were investigated further for characterization experiments.

Comparison of Supplements: Proliferation Assay and Cell Size

Primary cells isolated from donor cornea ($n = 3$ donors) were seeded in a 96-well plate at a density of 1000 cells/well. Images of each well were acquired every 2 hours for a total time period of 120 hours using an IncuCyte ZOOM (EssenBioScience, Ann Arbor, MI, USA). In total, 13 different combinations of basal medium and supplements were monitored for cell proliferation (Table 2). Population doubling time (PDT) was calculated using the following formula: $PDT = t \times \left[\frac{\log_{10}2}{(\log_{10}N_t - \log_{10}N_0)}\right]$ where N_0 is the initial

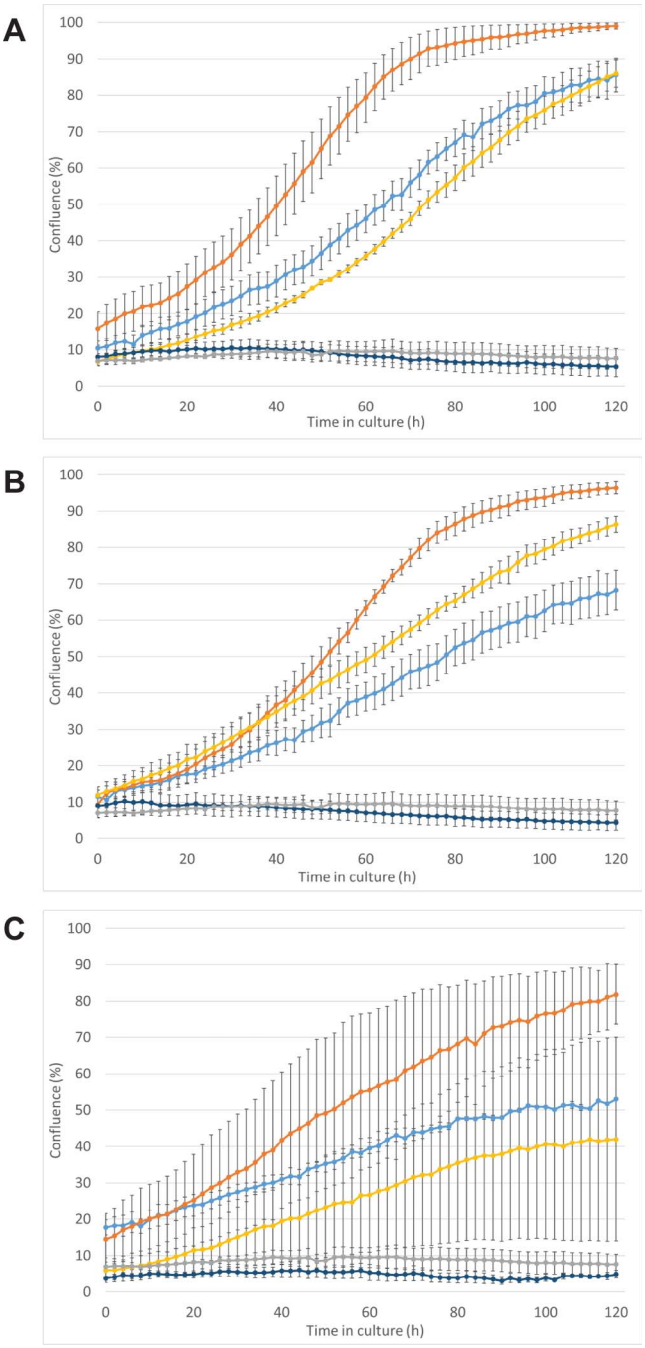

FIGURE 1. Overview of time to confluence for all cultures with a 10% supplement of either FBS, HPL, HAB, or XF (**A**), 5% supplement of either FBS, HPL, HAB, or XF (**B**), or 2.5% supplement of either FBS, HPL, HAB, or XF (**C**). $N = 3$ donors in triplicate per condition. Basal DMEM medium with no supplementation served as control. *Error bars*: standard deviation.

number of cells and N_t is the number of cells at a specific time point t (in hours). Cell size (area) of MSCs expanded in basal medium + 10% FBS and + 10% HPL was measured using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Calibrated images were acquired using the ×10 magnification objective in the IncuCyte ZOOM at different optical fields in the cell culture plate. Outlines of individual cells were traced and the surface area in square micrometers was calculated by ImageJ.

MSC Characterization: Flow Cytometry

Immunophenotypic analyses were performed using early passage cells (P4 or lower) from three donors cultured in either 10% FBS or 10% HPL. The cells used in this experiment were cultured in said media from the point of isolation onward. The cells were analyzed for the ISCT criteria using the following antibodies (all mouse anti-human and purchased from Becton Dickinson, Erembodegem, Belgium, unless stated otherwise): CD73-FITC (Antibody Registry: AB_10894209), CD90-FITC (AB_395969), CD105-PE (AB_868769; Abcam, Cambridge, UK), CD19-FITC (AB_1109962), CD34-FITC (AB_1953314), CD79α-PE (AB_2629278; Abcam), CD11b-PE (AB_395789), CD14-FITC (AB_1109783), CD45-FITC (AB_395874), and HLA-DR-PE (AB_2629277). The panel was extended to include additional specific antibodies frequently reported as being positively expressed on bone marrow MSCs[15]: CD13-PE (AB_400355), CD29-PE (AB_395836), CD44-FITC (AB_395870), and CD166-PE (AB_397210) (all from Becton Dickinson). 7-AAD (Becton Dickinson) was used to exclude dead cells. Samples were measured on the Becton Dickinson FACS scan and analyzed using FlowJo version 10 (FlowJo LLC, Ashland, OR, USA).

MSC Characterization: Trilineage Differentiation

The ability of corneal stromal hMSCs to differentiate into adipocytes, chondrocytes, and osteocytes was assessed using StemPro adipogenesis/osteogenesis/chondrogenesis differentiation media (Thermo Fisher Scientific, Waltham, MA, USA). Early passage cells originating from three donors cultured in both basal medium + 10% FBS and basal medium + 10% HPL were seeded in triplicate in a 48-well plate per differentiation lineage. Seeding densities, expansion time, and immunohistologic stainings for each lineage were executed as prescribed in the manufacturer's protocol. Lipid droplets signifying adipogenesis were identified using Oil Red O after 14 days of differentiation. Calcium deposits typical for osteogenesis were identified with Alizarin Red following 21 days of differentiation. Cartilage formation was detected using Alcian Blue after 14 days of differentiation. Cells undergoing chondrogenesis form cartilage micromasses, and the number of micromasses was tabulated per well.

RESULTS

Proliferation Assay and Cell Size

Detailed growth curve analyses of primary MSC cultures were performed to determine the influence of the supplement protocols. At a starting number of 1000 cells seeded per well, 100% confluence (plateau phase) was reached after 80 hours with 10% HPL. At the same time point, cells in parallel cultures with 10% FBS had a maximal confluence of just under 70%. Cultures with 10% HAB achieved only 60% confluence after 80 hours. XF-supplemented cultures showed no significant proliferation and attained 10.5% confluence over the total course of the experiment, comparable to performance of cells cultured in only the DMEM basal medium. The experiment concluded at 120 hours with only 10% HPL reaching the plateau phase (Fig. 1A). Cultures with 5% HPL began entering the plateau phase at around 90 hours (Fig. 1B). There was no significant difference in proliferation between 5% HPL- and 10% HPL-supplemented cultures. HAB supplemented at 5%

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Table 3. Mean Population Doubling Times in Hours

Supplement	Mean PDT, h ± SD
HPL 10%	21.2 ± 7.4
HPL 5%	24.6 ± 8.1
HPL 2.5%	27.3 ± 17.8
FBS 10%	32.1 ± 11.8
FBS 5%	36.5 ± 5.5
FBS 2.5%	38.1 ± 7.4
HAB 10%	35.1 ± 6.6
HAB 5%	42.1 ± 6.8
HAB 2.5%	49.1 ± 9.3
XF 10%	309.7 ± 109.5
XF 5%	319.2 ± 91.0
XF 2.5%	343.0 ± 144.0
No supplement	316.3 ± 225.3

showed higher proliferation than FBS supplemented at 5%. In 2.5% cultures the plateau phase was not reached within the time frame of the experiment (Fig. 1C). PDTs for all conditions are given in Table 3. Corneal MSCs cultured in 10% HPL showed significantly ($P < 0.001$) smaller cell surface area (3654 ± 208 μm^2, $n = 84$) compared to 10% FBS cultured corneal MSCs (5425 ± 406 μm^2, $n = 82$); see Figure 2.

Flow Cytometry

Both 10% FBS and 10% HPL showed the surface marker expression profile typical of MSCs in compliance with the ISCT requirements. Surface marker expression of CD73, CD90, and CD105 (Fig. 3) was detected in all cultures (>97% regardless of supplement); and despite measurable donor variation, no significant differences were observed. CD11b, CD19, CD14, CD34, CD45, CD79α, and HLA-DR all showed less than 1.5% positivity. Additional markers CD13, CD29, CD44, and CD166 (non-ISCT[25]) were all positively expressed (>92%) in all cultures (Table 4). No statistically significant differences were detected for either condition (*F*-test for equality of variances followed by paired 2-sample *t*-test assuming equal/unequal variances).

Trilineage Differentiation

MSCs derived from both 10% FBS- and 10% HPL-supplemented cultures demonstrated differentiation into the osteogenic, adipogenic, and chondrogenic lineage as demonstrated by positive Alizarin Red, Oil Red O, and Alcian Blue staining, respectively (Fig. 4). FBS-cultured MSCs formed significantly fewer micromasses per well (1.6 ± 0.4) than the HPL-grown MSCs (3.6 ± 0.5) (Student's *t*-test, $P = 0.02$).

Discussion

In this study we examined four supplements at three different concentrations and unsupplemented basal medium, giving rise to a comprehensive comparison of 13 conditions in total, which were tested for primary MSCs isolated from human donor corneas. Our results show that the rate of expansion of corneal stromal MSCs is significantly higher with 10% HPL compared to none, XF, HAB, or FBS (added at 2.5%, 5%, or 10%) based on confluence levels of the cultures. There was no significant difference in proliferation between 5% and 10% HPL-supplemented cultures, indicating that for future applications the HPL content in MSC cultures could be reduced although further optimization is required. When supplemented at 5%, HAB serum outperformed FBS. This was not observed

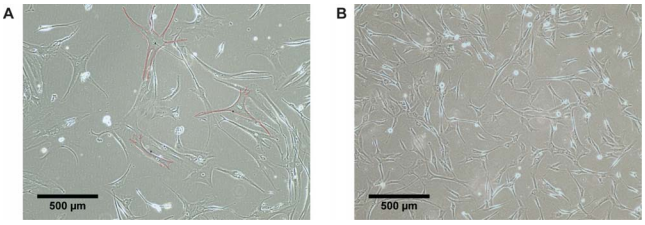

Figure 2. Corneal MSC cultures in DMEM + 10% FBS (**A**) and DMEM + 10% HPL (**B**), magnification ×10.

for cultures supplemented at 10% or 2.5%, where the confluence of FBS-supplemented cultures was higher than that of HAB-supplemented cultures.

Mean PDTs (Table 3) show that cells cultured in HPL grow approximately 1.5 times faster than cells cultured in FBS, regardless of being supplemented at 2.5%, 5%, or 10%. Confluence was achieved faster in HPL compared to FBS cultures despite the significantly smaller size of MSCs grown in HPL ($P < 0.0001$). HPL-grown cells are on average 30% smaller in size than FBS-grown cells, and together with the lower PDT (21.2 ± 7.4 hours for 10% HPL-supplemented cultures versus 32.1 ± 11.8 hours for 10% FBS-supplemented cultures), this shows that confluence was achieved earlier due to faster proliferation and not due to enlargement of the cells in HPL cultures. Christodoulou et al.[26] have previously shown that there is a positive correlation between smaller cell size and low PDT for fetal Wharton's Jelly MSCs and adipose tissue–derived MSCs. PDTs of hMSCs (Table 3), grown without supplement, with XF (2.5%, 5%, and 10%), or with any of the other supplements at 2.5%, were calculated, but the relevance of these values can be questioned due to the lack of exponential growth in these cultures.

Subsequent cultures of MSCs grown in either 10% FBS or 10% HPL showed no significant differences in expression of the ISCT required marker panel or in additional markers tested (typical MSC-positive markers), and thus it was concluded that the phenotype of MSCs grown in 10% FBS is comparable to the phenotype of MSCs grown in 10% HPL. Furthermore, it was observed that HPL-supplemented cultures consistently surpassed FBS ones with regard to the growth rate of corneal stromal MSCs, reaching confluence well in advance of FBS cultures. Differentiation experiments were successful for all three lineages in each of the donors tested for both 10% FBS- and 10% HPL-supplemented cultures, and the differentiation potential was deemed equal. In chondrogenic differentiation medium it was observed that 10% HPL-cultured MSCs formed significantly more micromasses than 10% FBS-cultured MSCs (*P* value 0.02).

Animal-free alternatives to FBS have been studied before, but all previous research has targeted MSC sources other than the corneal stroma, with almost all research being focused on MSCs sourced from the human bone marrow. These earlier studies suggested HPL as a suitable xeno-free alternative to FBS for hMSC expansion.[21,27–29] FBS and HPL are both suitable supplements for a wide range of cell types; they are rich in growth factors, but HPL is inherently richer in growth factors from the platelet fraction. It has been shown that HPL stimulates a faster growth, specifically that of MSCs, and that there is no risk of xenogeneic immune reactions or disease transmission with a potential for autologous applications.[18] Genomic stability of MSCs cultured in HPL has been shown for adipose and bone marrow hMSCs.[30] These are some major advantages over the current gold standard, FBS.

The main disadvantage to the use of HPL is the possible variability in HPL between suppliers. This variability can be

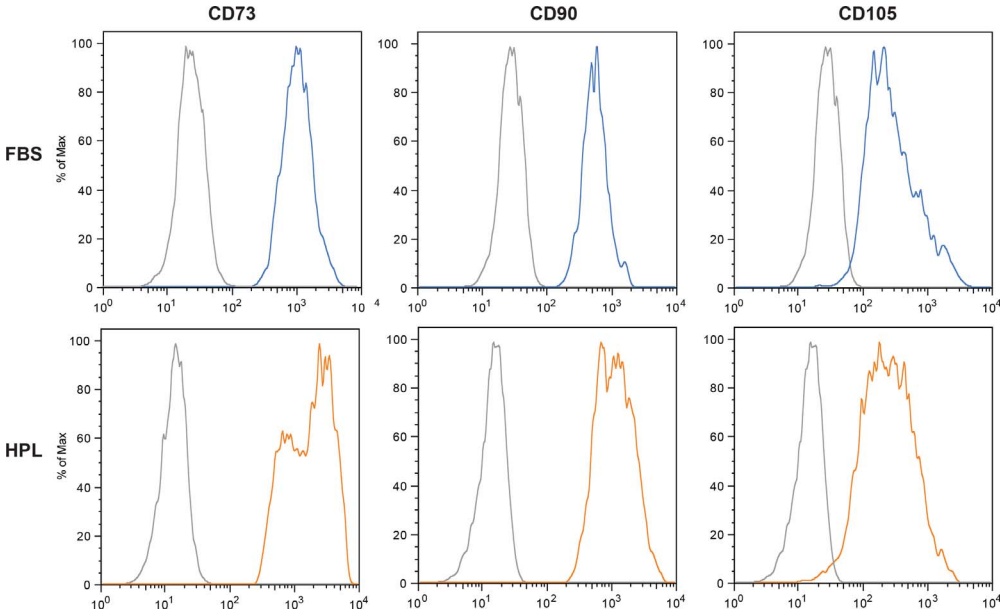

Figure 3. hMSCs isolated from the corneal stroma are positive for CD73, CD9,0 and CD105. Isotype controls are shown as *gray curves* and specific markers are shown by *blue* (FBS-supplemented cultures) and *orange* (HPL-supplemented cultures).

introduced in several steps in the production process: Platelets can be obtained from apheresis or whole blood donation, with or without leukoreduction; heparin may be added to avoid clotting; a variable number of donors can be pooled; and manufacturers are making use of either expired or nonexpired platelet concentrates.[31,32] Although repeated freezing and thawing is the most commonly used method to obtain platelet lysates, other options include direct activation using calcium chloride or thrombin, sonication, and solvent/detergent treatment, depending on the manufacturer.[31,32]

HPL is commercially available as an allogeneic off-the-shelf product, but it can also be produced as an autologous supplement. Human MSCs from bone marrow have been shown to proliferate faster in autologous HPL-supplemented than in allogeneic HPL-supplemented cultures.[33] Allogeneic HPL is manufactured on a large scale and is therefore a cost-effective option with limited variation in growth factor content from batch to batch, while on the other hand there can be a risk of alloimmunization. Autologous HPL does not entail the risks of immunization but is prone to lot-to-lot variability; and because only small volumes can be produced it is much less interesting in terms of cost-effectiveness. Regardless of the autologous or allogeneic origin of the HPL, many studies on bone marrow– and adipose-derived MSCs have shown the superiority of HPL over FBS regardless of the supplier.[30,32]

Table 4. Overview of Marker Expression With Standard Deviation (%), $N = 3$ Donors

Criterion	Marker	10% FBS	10% HPL
	CD73	99.07 ± 1.20	99.10 ± 0.54
ISCT positive	CD90	99.83 ± 0.05	99.73 ± 0.12
	CD105	99.60 ± 0.28	99.63 ± 0.24
	CD11b	0.20 ± 0.14	0.14 ± 0.01
	CD14	0.90 ± 0.37	0.14 ± 0.01
	CD19	0.70 ± 0.42	0.07 ± 0.05
ISCT negative	CD34	0.37 ± 0.05	0.13 ± 0.12
	CD45	0.10 ± 0.00	0.10 ± 0.08
	CD79α	0.10 ± 0.00	0.07 ± 0.05
	HLA-DR	0.33 ± 0.17	0.13 ± 0.05
	CD13	98.03 ± 1.48	96.13 ± 2.67
Non-ISCT positive	CD29	99.63 ± 0.31	99.73 ± 0.17
	CD44	99.70 ± 0.00	98.87 ± 0.50
	CD166	92.80 ± 5.40	93.43 ± 5.27

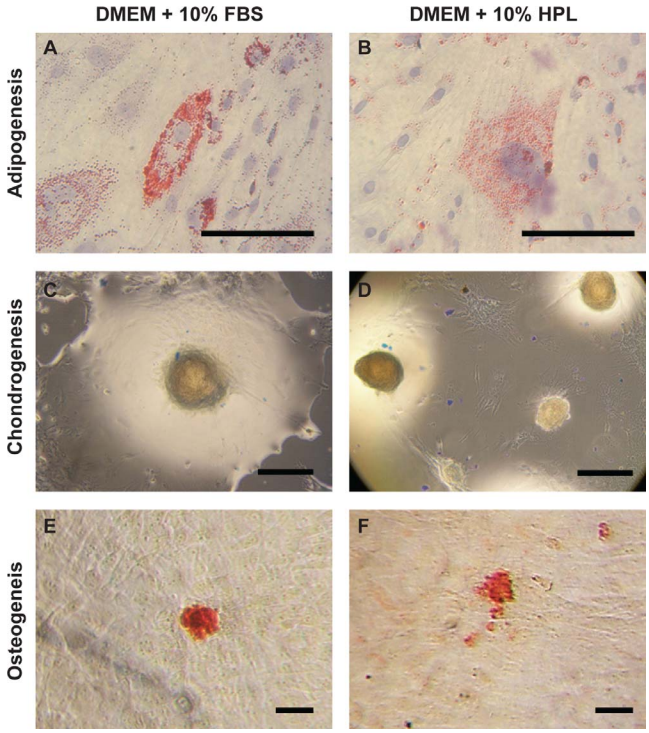

Figure 4. Corneal stromal MSCs cultured in DMEM + 10% FBS versus 10% HPL were differentiated to adipogenic (**A**, **B**), chondrogenic (**C**, **D**), and osteogenic lineages (**E**, **F**), stained using Oil Red O (adipogenesis), Alcian Blue (chondrogenesis), and Alizarin Red (osteogenesis). $N = 3$ donors in triplicate per lineage. *Scale bars*: 500 μm.

Our results confirm the conclusions of earlier studies, but for the first time the newly discovered corneal stromal MSCs were targeted. A comparative investigation of FBS alternatives for MSC expansion and the effects on stemness has not been performed in the past for this specific cell type.

In conclusion, one of the key challenges that remains for both drug discovery as well as clinical applications is obtaining a sufficient number of cells at a reasonable cost.[34] With the advent of clinical trials investigating improved corneal stromal wound healing[35] and prevention of scarring, research into scaling up production of this particular cell type becomes imperative. The added advantage of the immunosuppressive nature of these cells means that future therapies could foresee administration of an off-the-shelf allogenic cell product, making research into high throughput production of clinically relevant cell numbers without a loss in quality highly relevant.[36] From our results we conclude that out of the 13 conditions tested, HPL supplemented at 10% generates the best growth kinetics for proliferating corneal stromal MSCs while maintaining their viability, cellular phenotype, and differentiation potential. HPL is therefore a better alternative to FBS for cultivation of corneal stromal MSCs, and should replace it as the new gold standard.

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