

Comparative Analysis of Media and Supplements on Initiation and Expansion of Adipose-Derived Stem Cells

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Key Words. Adipose-derived stem cells • Culture media • Xenogeneic free • Cell proliferation • Phenotype • Cell therapy

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Received July 7, 2015; accepted for publication November 2, 2015; published Online First on February 2, 2016.

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1066-5099/2016/\$20.00/0

<http://dx.doi.org/10.5966/sctm.2015-0148>

ABSTRACT

Adipose-derived stem cells (ASCs) are being tested in clinical trials related to cell-based regenerative therapies. Although most of the current expansion protocols for ASCs use fetal calf serum (FCS), xenogeneic-free medium supplements are greatly desired. This study aims to compare the effect of FCS, human platelet lysate (hPL), and a fully defined medium on the initiation and maintenance of ASC cultures. ASCs obtained from five donors were cultured in five different media: StemPro, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% hPL, or α -minimum essential medium (A-MEM) supplemented with 5% hPL, 10% hPL, or 10% FCS. The effect of media on proliferation, colony-forming units (CFUs), attachment, and morphology was assessed along with cell size, granularity, and immunophenotype. StemPro greatly compromised the initiation of ASC cultures, which could not survive more than a few passages. Cells cultured in A-MEM proliferated at a faster rate than in DMEM, and hPL significantly enhanced cell size, granularity, and proliferation compared with FCS. All media except StemPro supported CFUs equally well. Analysis of surface markers revealed higher levels of CD73 and CD105 in FCS-cultured ASCs, whereas increased levels of CD146 were found in hPL-cultured cells. Multiparametric flow cytometric analysis performed after seven passages revealed the existence of four distinct ASC subpopulations, all positive for CD73, CD90, and CD105, which mainly differed by their expression of CD146 and CD271. Analysis of the different subpopulations might represent an important biological measure when assessing different medium formulations for a particular clinical application. *STEM CELLS TRANSLATIONAL MEDICINE* 2016;5:314–324

SIGNIFICANCE

In most clinical trials using adipose-derived stem cells (ASCs), the cells have been expanded in culture media supplemented with fetal calf serum. However, there is much interest in replacing fetal calf serum with human platelet lysate or using completely serum- and xenogeneic-free media. This study found that culture in fetal calf serum versus human platelet lysate had a significant effect on the degree of expression of stem cell-associated surface markers. These results underscore the need to carefully investigate the effect of culture media on ASC behavior before committing to one medium type for clinical use.

INTRODUCTION

Adipose-derived stem cells (ASCs) are a heterogeneous population of adult multipotent stem cells that can be isolated with relative ease and in substantial amounts from the stromal vascular fraction (SVF) of adipose tissue using the protocol of Zuk et al. [1]. In a joint statement by the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy, it was proposed that ASCs should be characterized by their capacity for plastic adherence; their ability to differentiate into bone, cartilage, and adipose tissue; and their expression of a selected set of surface

markers including CD73, CD90, and CD105 [2]. On review of 15 recent clinical trials on ASCs, however, we found that in a majority of trials, ASCs have been characterized based on a limited set of surface markers, most often including CD73, CD90, and CD105, and in only one trial was trilineage differentiation potential assessed (supplemental online Table 1).

Initially, the multilineage potential of ASCs garnered much attention, as it was proposed that ASCs could be used for ex vivo tissue engineering of osteochondral tissue; indeed, in animal models and human case reports, it has been demonstrated that ASCs may be used to construct functional

tissue, such as bone and trachea, that is suitable for transplantation [3, 4]. However, recent years have seen a shift in focus, and today ASCs are increasingly being recognized as having strong regenerative capacity because of their paracrine activity facilitated by soluble mediators, immunomodulatory properties, and capacity to home to injured tissues [5]. This shift has increased their attraction, as they now are seen not as building blocks but as initiators and regulators of tissue regeneration. Research on the potential clinical usefulness of ASCs, for example in cardiac regeneration, has moved from promising *in vitro* and animal studies [6–8] to clinical trials [9, 10]. In addition to these studies, clinical trials are completed or under way for the use of ASCs to treat critical limb ischemia [11], perianal fistula [12], Crohn's fistula [13, 14], osteoarthritis [15], and acute respiratory distress syndrome [16], to name but a few.

For most if not all clinical indications, after the isolation of SVF, cells must be expanded *in vitro* to achieve the necessary therapeutic dose [17, 18], which also results in a more homogeneous and less immunogenic product [17]. Optimal cell growth depends on a variety of microenvironmental factors including oxygen tension in the culture system [6, 19–22], topography of the culture surfaces [23, 24], mechanical factors [25, 26], and most notably, cell culture medium composition [27, 28]. Basically, the culture medium has to supply all essential nutrients, hormonal factors, transport proteins, minerals, lipids, attachment and spreading factors, and stabilizing and detoxifying factors to reach optimal cell metabolism, growth, and proliferation [29]; often medium compositions that promote maximal proliferation are perceived as superior.

For historical, practical, and economic reasons, ASCs are typically expanded in basal medium supplemented with fetal calf serum (FCS), and *ex vivo* expanded ASCs have been used in several clinical trials [9, 13, 14, 30–35]. However, even Good Manufacturing Practice-approved FCS is a complex mixture of essential nutrients and bioactive molecules with high lot-to-lot variability. Furthermore, there is concern that the use of FCS increases the risk of contamination and immunization because of the presence of xenogeneic components and possibly viruses, which can lead to transplantation failure [36–40]. To address this issue, it has been proposed that human blood-derived products, such as autologous platelet-derived products [41, 42], should be used; however, apart from being an impractical solution, advanced donor age has been shown to have a negative effect on the multipotential of mesenchymal stem cells (MSCs) [43]. The use of allogeneic human platelet lysate (hPL) has been proposed as an alternative to FCS and has been shown to be a safe option [29, 44]. hPL is generated from common platelet units by a simple freeze-thaw procedure and contains a variety of growth factors, proteins, and enzymes to support attachment, growth, and proliferation; yet it is still poor in antibodies and has been shown to promote the growth of various cell lines including ASCs [38, 44]. Furthermore, it has been convincingly documented that during and after expansion in medium supplemented with hPL, ASCs retain their capacity for trilineage differentiation [45, 46]. Potential caveats on the use of hPL are that it is not fully defined as a supplement, there are lot-to-lot variations, and there is a risk of pathogen carryover, albeit small.

To overcome these disadvantages, chemically defined, serum-free, and xenogeneic-free media have been developed. Because the growth factors essential for mesenchymal stem cell (MSC) isolation and expansion have not yet been identified, several synthetic media have proven unsuitable for ASC expansion; they have been shown to support ASC proliferation and at the same time fail to support ASC properties in terms of differentiation potential [28, 47].

Novel synthetic media, however, show more promise than the earlier types in terms of promoting proliferation while maintaining multipotentiality, and we and others have previously shown that expansion of ASCs in synthetic serum- and xenogeneic-free StemPro media supports trilineage differentiation [27, 48].

In this study, we compared culture in media supplemented with hPL, FCS, and synthetic media with respect to ASC isolation from SVF and expansion in terms of attachment, colony-forming capacity, growth rate, and immunophenotype. We found that synthetic medium was unsuitable for initiation of ASC cultures from SVF, and that whereas culture in either FCS or hPL yielded ASCs displaying the characteristic phenotype defined by positive expression of surface markers CD73, CD90, and CD105, there was a difference between the two supplements in the degree to which the individual ASCs expressed several stem cell-associated surface markers.

MATERIALS AND METHODS

Isolation and Culture of ASCs

For harvesting ASCs, excess subcutaneous adipose tissue was obtained from five donors undergoing elective liposuction surgery at Teres Private Hospital, Skejby, Denmark. All patients gave written informed consent, and the protocol was approved by the regional committee on biomedical research ethics of Northern Jutland, Denmark (project no. VN 2005/54). A summary of patient data is found in supplemental online Table 2. This study complied with the principles of the Declaration of Helsinki.

ASCs were isolated according to methods previously described by our laboratory [49], with minor modifications. Briefly, adipose tissue was washed repeatedly with sterile phosphate-buffered saline (PBS) (Invitrogen, Taastrup, Denmark, <https://www.thermofisher.com/>) and then dissociated enzymatically for 60 min at 37°C with 0.6 U/ml Collagenase NB 4 Standard Grade (Serva Electrophoresis, Heidelberg, Germany, <http://www.servade.com/>) in Hanks balanced salt solution (HBSS; Invitrogen) under continuous, gentle agitation. The SVF within the dissociated tissue was filtered through a 100- μ m filter and pelleted by centrifugation at 400g for 10 min. The pellet was resuspended and filtered through a 60- μ m filter and pelleted again by centrifugation at 400g for 10 min, forming the SVF. The cells were resuspended in PBS, and the cell yield was determined with a Nucleocounter NC-200 cell counter (Chemometec, Allerød, Denmark, <http://chemometec.com/>). Cells were divided into aliquots to allow for parallel experiments with different media. The culture media were α -minimum essential medium (A-MEM) with GlutaMAX (Invitrogen) supplemented with 10% FCS (Invitrogen), A-MEM supplemented with 10% hPL (Stemulate; Cook Medical, Bloomington, IN, <https://www.cookmedical.com/>), A-MEM supplemented with 5% hPL, Dulbecco's modified Eagle's Medium (DMEM) with GlutaMAX (Invitrogen) supplemented with 10% hPL, or StemPro MSC SFM XenoFree (Invitrogen) supplemented with L-glutamine (Invitrogen). They were all supplemented 100 U/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen) and cultured on tissue culture propylene (TCP; Greiner Bio-One, Fredensborg, Denmark, <http://www.greinerbioone.com/>). Explanations of medium abbreviations are given in Table 1. The tissue culture surface for the cells cultured in StemPro were additionally coated with CellStart CTS (Invitrogen) according to the manufacturer's protocol. Because of limitations of the resulting SVF cell number, parallel cultures of at most four different culture media were possible for each of

Table 1. Compositions of the different media used in this study

Medium	Description	Donors, <i>n</i>
A-MEM ^{FCS}	α -Minimum essential medium with GlutaMAX + 10% fetal calf serum	3
A-MEM ^{hPL5}	α -Minimum essential medium with GlutaMAX + 5% human platelet lysate	5
A-MEM ^{hPL10}	α -Minimum essential medium with GlutaMAX + 10% human platelet lysate	5
DMEM ^{hPL10}	Dulbecco's modified Eagle's medium with GlutaMAX + 10% human platelet lysate	2
StemPro	StemPro MSC SFM XenoFree	3

Abbreviations: A-MEM, α -minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; hPL5, 5% human platelet lysate; hPL10, 10% human platelet lysate; SVF, stromal vascular fraction.

the five donors (Table 1). To compensate for interdonor variations and facilitate comparisons between all media, A-MEM^{hPL5} and A-MEM^{hPL10} were included in the experimental setups for each donor. The abbreviation SVF is used throughout the study for cells not yet passaged, and the term ASC denotes cells after first passage.

Proliferation

To determine the effect of medium composition on the proliferation rate of ASCs, SVFs were seeded at a density of 150,000 cells per cm² in T25 Cellstar Tissue Culture Flasks (Greiner Bio-One), and after overnight incubation, thoroughly washed with PBS to remove unattached cells. ASCs were cultured in a standard Steri-Cycle CO₂ incubator in a humidified atmosphere containing 20% O₂ and 5% CO₂ at 37°C, with medium changes twice a week. When the first of the parallel cultures reached 80% confluence, all cultures were subcultured using TrypLe (Invitrogen), and the number of ASCs per flask was counted using a hemocytometer. The cells were cultured for up to four passages in which ASCs were seeded at a density of 2,000 cells per cm² in T25 tissue culture flasks, maintained with medium changes twice a week, and passaged and counted when the first culture reached 80% confluence. Cultures of SVFs were performed in quadruplicate and ASCs in triplicate for each donor. Accumulated cell number (*N*) was calculated from $N(t) = c \cdot 2^{t/d}$, where *N*(*t*) is the number of cells at time *t*, *d* is doubling time, and *c* is total number of cells after previous passage. Doubling time was calculated from the following: $t \cdot \log(2)/\log(e/s)$, where *e* is harvested number of cells and *s* is seeding density. Population doubling was calculated for each passage according to the equation $PD = 3.32(\log X_e - \log X_b)$, where *X_e* is the cell number at the end of the passage and *X_b* is the cell number at the beginning of the passage [50].

Attachment

The effect of the different media on cell attachment was assessed by seeding SVFs at a density of 150,000 cells per cm² and ASCs in passages 1 and 2 at 2,000 cells per cm² in T25 Cellstar Tissue Culture Flasks, incubating overnight, and thoroughly washing the culture vessels with PBS to remove unattached cells. After washing, phase-contrast pictures were taken at three random locations for each culture flask using an Olympus CKX41 microscope (Olympus

Life Science Solutions, Chelmsford, MA, <http://www.olympus-lifescience.com>) with a PixeLINK PL-A782 camera. The number of cells attached to the culture surface was determined for a fixed surface area for each picture.

CFUs

To determine the frequency of colony-forming units (CFUs) in the cultures grown in the different medium compositions, SVFs and ASCs were seeded in limiting dilution in a 96-well plate (Costar, Corning Life Sciences, Tewksbury, MA, <http://www.corning.com>) according to Schellenberg et al. [51] with minor changes. In brief, ASCs were seeded at densities of 10–1,000 cells per well for SVF and 1–30 cells per well for ASCs in passages 1–3 and cultured with medium changes twice a week. At day 12, the cells were fixed in 10% formalin (Ampliqon, Odense, Denmark, <http://www.ampliqon.com/>) and stained using 0.05% Crystal Violet (Sigma Aldrich Denmark, Brøndby, Denmark, <http://www.sigmaldrich.com/denmark/om-danmark.html>) in H₂O. Wells containing one or more colonies were noted, and L-Calc Software (StemCell Technologies, Vancouver, BC, Canada, <http://www.stemcell.com/>) was used to calculate the proportion of CFUs using Poisson statistics. Data not following the Poisson distribution were excluded from analysis.

Surface Marker Phenotypic Analysis

Flow cytometric analysis was performed on a MOFLO Astrios EQ (Beckman Coulter, Birkerød, Denmark, <https://www.beckmancoulter.com>) using a six-color stain setup that included the following antibodies (BD Bioscience, Albertslund, Denmark, <http://www.bd.com>) and channels: anti-CD73 fluorescein isothiocyanate on 488-513/26, anti-CD90 peridinin chlorophyll protein-cyanine 5.5 on 488-710/45, anti-CD105 allophycocyanin on 640-664/22, anti-CD146 phycoerythrin-CF594 on 561-614/20, and anti-CD271 phycoerythrin-cyanine 7 on 561-795/70. Analysis of the culture surface marker phenotype was based on SVFs collected immediately after isolation and ASCs collected at passages 1 and 7 grown in the five different medium compositions. Before staining the SVFs, erythrocytes were lysed in distilled water for 10 seconds, after which a physiological salt balance was restored. Before, between, and after staining, cells were washed in staining buffer (0.1% bovine serum albumin in PBS). The cells were first stained with the five antibodies for 30 min and afterward stained with Live/Dead Fixable Aqua Dead Cell Stain (Molecular Probes, Taastrup, Denmark, <http://www.thermofisher.com>) for another 30 min. Next, the cell sample suspended in staining buffer was dissociated using a cell strainer-capped tube to ensure single cells (BD Falcon; BD Biosciences, Erembodegem, Belgium, <http://www.bdbiosciences.com>). Stream alignment was optimized using Sphero Ultra Rainbow Fluorescent Particles (Spherotech, Lake Forest, IL, <http://www.spherotech.com/>). The voltage and gains of the photomultiplier tubes were adjusted inside the linear range so that an unstained sample produced a bell-shaped emission with a median fluorescence of 10¹ and no double-negative peaks apparent in the red channels. Compensations were made with BD CompBeads Set Anti-Mouse Ig, κ (BD Bioscience) to standardize compensation despite differences in marker expression and to ensure sufficient bright populations when markers were vaguely expressed. The resulting compensation matrix was individually fitted to each sample based on dot-plots depicting the combination of channels in question.

Table 2. Effect of media on expression of surface markers

Marker	SVF	A-MEM ^{FCS}		A-MEM ^{hPL5}		A-MEM ^{hPL10}	
		Passage 1	Passage 7	Passage 1	Passage 7	Passage 1	Passage 7
CD73 ⁺	53.5 ± 17.5	92.3 ± 6.6	99.7 ± 0.3	90.4 ± 6.5	99.3 ± 0.4	92.1 ± 6.7	99.2 ± 0.7
CD90 ⁺	82.3 ± 14.6	97.6 ± 1.2	99.7 ± 0.2	97.8 ± 1.2	99.9 ± 0.1	97.9 ± 1.3	100.0 ± 0.0
CD105 ⁺	14.0 ± 12.1	98.7 ± 0.9	98.1 ± 1.9	98.7 ± 0.8	95.5 ± 4.3	99.2 ± 0.5	92.4 ± 9.7
CD146 ⁺	21.3 ± 2.7	52.1 ± 6.7	48.7 ± 8.4	32.3 ± 9.3	38.3 ± 12.5	41.6 ± 28.5	54.0 ± 26.4
CD271 ⁺	11.1 ± 4.8	16.9 ± 0.7	10.7 ± 0.6	31.5 ± 25.5	3.0 ± 2.1	23.5 ± 18.1	6.8 ± 5.6

Results are presented as mean ± SD percentage of positive cells for each marker.

Abbreviations: A-MEM, α -minimum essential medium; FCS, fetal calf serum; hPL5, 5% human platelet lysate; hPL10, 10% human platelet lysate; SVF, stromal vascular fraction.

Fluorescence minus one (FMO) controls were included for each fluorochrome, donor, and passage. Flow-Set Pro Fluorospheres (Beckman Coulter) were analyzed in regard to coefficient of variation and mean fluorescence intensity with each sample to ensure sample comparability. Cell viability was assessed on 355-448/59. Data were visualized and analyzed using Kaluza version 1.3 (Beckman Coulter). Cells were first gated on a forward scatter (FSC) (height)/side scatter (height) contour plot to gate out noise and debris, then a FSC (width)/FSC (height) contour plot was used to discriminate doublets. Subsequently, viable cells were gated out. The resulting cells were then plotted in histograms corresponding to the above markers, and overlays between FMOs and the stained sample were created. Positivity was defined by an overlay marker whose lower boundary was set to include the top 2.5% of the FMO.

Statistical Analysis

All data have been normalized to the level of A-MEM^{hPL10} at first passage for each individual donor because of large interdonor variation. All statistical analysis was performed using SigmaPlot 12.0 (Systat Software, Erkrath, Germany, <http://www.systat.com>). The normal distribution of each group was assessed by means of the Shapiro-Wilk test. Additionally, variance was tested using an equal variance test. Data are represented as mean ± SEM, except for the quantitative flow cytometry data, where data are presented as mean ± SD. A *p* value < .05 was considered statistically significant. For comparison of two groups, a Student *t* test or a Mann-Whitney rank sum test was used. When comparing more than two groups, one-way analysis of variance with Bonferroni's post hoc test was used.

RESULTS

Effect of Medium Composition on Establishment of ASC Cultures

To determine the effect of medium composition on cell growth, freshly isolated cells were cultured for up to three passages in different media. From visual inspection of the cultures, it was apparent that media supplemented with hPL supported growth of ASCs better than either StemPro or media supplemented with FCS (Fig. 1A). Quantitative assessment of cell growth for up to four passages confirmed the superiority of hPL as a medium supplement and revealed a significantly higher cumulative number of cells in all three media supplemented with hPL at the end of passages 2, 3, and 4 compared with A-MEM^{FCS} (Fig. 1B). Data are expressed on a log 10 scale to optimally visualize the exponential growth.

As explained in the section on statistical analysis, the cell numbers are normalized and therefore are expressed as percentages. The higher cumulative cell number is a result of shorter population doubling time (supplemental online Fig. 1). Most of the media supported stable cell growth during serial passages (Fig. 1C). Interestingly, however, StemPro did not consistently support cell growth, and we observed that for two of three donors, the corresponding cell cultures could not be satisfactorily maintained for more than three passages, and for the third donor, no more than five passages.

The effect of the media on cell attachment and support of CFUs was determined for SVFs and ASCs in early passages (Fig. 2). Overnight culture of SVFs and subsequent removal of nonadherent cells resulted in mixed populations of mostly spheric and a few slightly elongated cells, with no obvious morphologic differences between cells grown in the different media (Fig. 2A). For all media, passaging (both passages 1 and 2) resulted in changes in cell morphology and spreading, with the cells looking larger and more spindle shaped. For cells in passages 1 and 2, it appeared that culture in StemPro promoted cell attachment. A quantitative measure of the cell attachment within 16 hours after seeding (Fig. 2B) confirmed that StemPro did promote cell attachment to a higher degree than the other media, in particular higher than the hPL-supplemented media. The DMEM-based media, on the other hand, consistently supported cell attachment to a lower degree than the other media.

To determine whether medium composition influenced the proportion of highly proliferative and clonogenic cells in the cultures, a CFU assay was performed (Fig. 2C). Not surprisingly, for all medium compositions the proportion of CFUs was higher in the passaged cells than in the SVFs. Also, comparing the frequency of CFUs across all passages, StemPro significantly underperformed, and there were no consistent differences between the other media. Furthermore, an obvious difference in colony size and confluence was observed, with the hPL-supplemented media being superior to both FCS and StemPro (data not shown).

Effect of Expansion in Different Media on ASC Morphology and Phenotype

The effect of expansion on cell size and granularity was determined by flow cytometry. Figure 3 displays the analysis of cells from a representative donor. Regardless of supplement, cells cultured in A-MEM for one passage were larger and more granular than the cells composing the SVFs (Fig. 3A). Also it appeared that A-MEM^{hPL5/10} promoted subpopulations of cells that were larger and with a more granular phenotype than A-MEM^{FCS}. At passage 7, this difference was even more prominent, with the cells cultured in A-MEM^{FCS} representing a smaller, less granular, and more

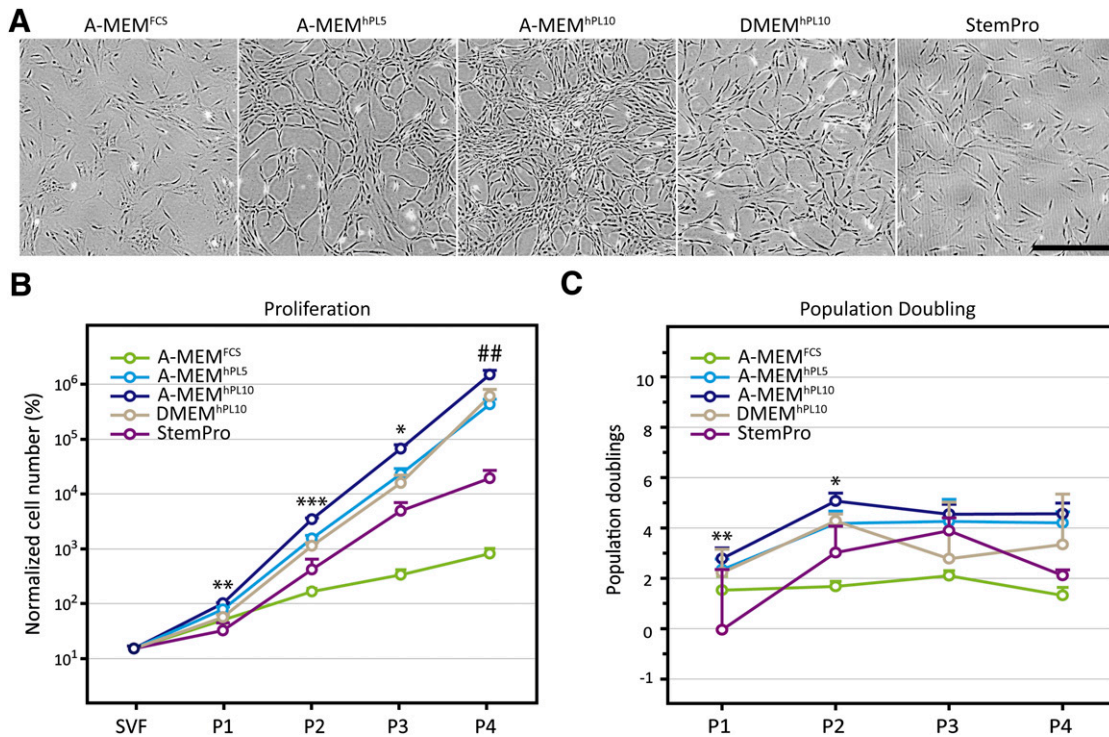


Figure 1. Assessment of proliferation of adipose-derived stem cells (ASCs). **(A):** ASC cultures reached different cell densities during expansion depending on media, as evident at passage 1 after 4 days of culture. **(B):** Accumulative cell number after a series of passages, where a clear effect of media is shown. **(C):** Number of population doublings that each population underwent during each passage. The same tendency as in **(B)** was seen. The results are presented as mean \pm SEM. Cell number was normalized to the cell number of each individual donor for A-MEM^{hPL10} at passage 1. A-MEM^{hPL10} was statistically different from all other media at $p < .05$ (*), $p < .01$ (**), and $p < .001$ (***), and statistically different from all other media except DMEM at $p < .007$ (##). Scale bar = 500 μ m. Abbreviations: A-MEM, α -minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; hPL5, 5% human platelet lysate; hPL10, 10% human platelet lysate; P, passage; SVF, stromal vascular fraction.

homogeneous population than cells in A-MEM^{hPL5} or A-MEM^{hPL10}. These observations were consistent across donors (Fig. 3B). Interestingly, whereas cells cultured in DMEM appeared to have characteristics similar to cells cultured in A-MEM, cells cultured in StemPro seemed less granular (supplemental online Fig. 2).

A qualitative assessment of selected surface markers showed that for the SVFs, >40% of the cells were positive for CD73 and CD90 for all donors (Table 2; supplemental online Table 3). As for the expression of CD105, on average only 14% of the cells were positive, and there was large interdonor variation. After just one passage in any A-MEM-based medium, >80% of the cells from all compositions were CD73⁺, CD90⁺, and CD105⁺. The average proportion of CD146⁺ and CD271⁺ cells in the SVF was relatively low, 21% and 11%, respectively. The average number of CD146⁺ cells increased with passaging to almost 50% at passage 7, and the amount of CD271⁺ cells decreased and almost disappeared for some donors.

A comparative analysis of surface marker levels revealed substantial differences between cells cultured in the different media. This is illustrated in Figure 4, which depicts the intensity levels for all analyzed markers from a representative donor. It was evident that the number of CD73⁺ cells increased with passaging, especially from SVF to passage 1. However, this effect was less pronounced for the cells cultured in StemPro. At passage 7, all cells were positive for CD73; interestingly, though, culture in FCS seemed to stimulate expression levels more than culture in hPL, resulting in a more bright CD73⁺ population in A-MEM^{FCS}. No differences between cells cultured in either 5% or 10% hPL were found. Also, we did not detect

a difference between cells cultured in A-MEM^{hPL} and DMEM^{hPL} (supplemental online Fig. 3). We observed no effect of media on the expression level of CD90. The expression of CD105 mirrored the profile of CD73, with the cells becoming increasingly positive with passaging, and with an even larger difference in the antigen expression level between cells cultured in either FCS or hPL. In contrast, regarding CD146 expression, although the proportion of bright cells decreased from passage 1 to passage 7, culture in A-MEM^{hPL10} appeared to maintain a relatively higher expression level. These tendencies were observed for all donors.

Figure 5 shows multiparametric analysis of the surface markers performed on cells from a representative donor. In the SVF sample, a very heterogeneous distribution with a variety of cell subpopulations was found. Populations with all combinations of the used markers were identified. The two largest subpopulations were CD90⁺, CD105⁻, CD146⁻, and CD271⁻ and then either CD73⁺ or CD73⁻, comprising 22% and 21% of the total cell number, respectively. At passage 1, cells cultured in StemPro retained relatively more of the heterogeneous phenotype. However, cells cultured in A-MEM^{FCS/hPL} were considerably more homogeneous. The CD105⁻ populations had almost disappeared, and the majority of the cells fell in two categories that were CD90⁺, CD105⁺, and either CD73⁺ or CD73⁻. At passage 7, the populations were even more homogeneous compared with passage 1, as the CD73⁻ populations were no longer found. Most of the cells were of the phenotype CD73⁺, CD90⁺, CD105⁺, CD271⁻, and either CD146⁺ or CD146⁻. This general

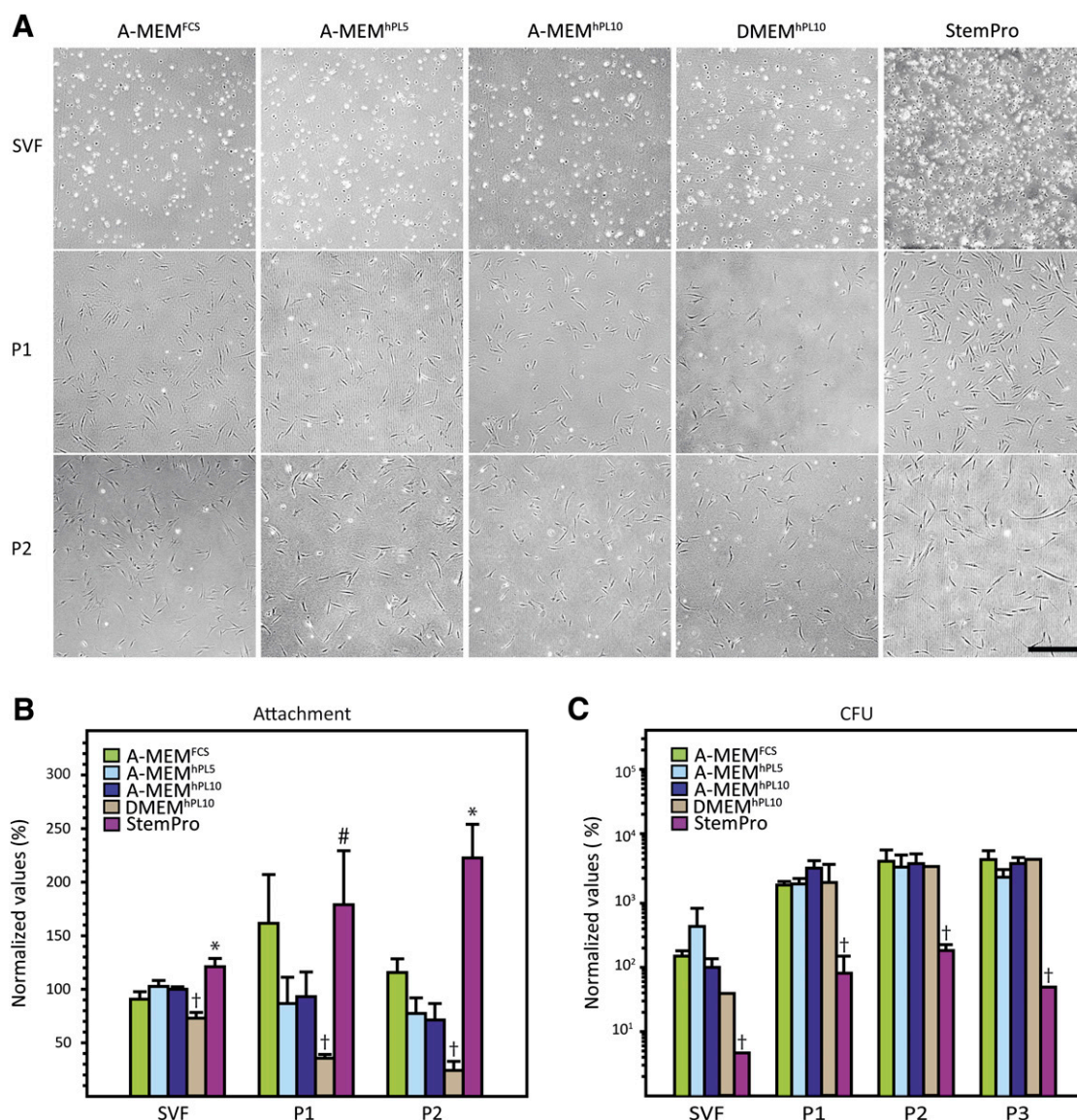


Figure 2. Cell morphology, attachment (A, B), and clonogenicity (C). (A): Differences in the degree of cell attachment were observed after seeding and overnight incubation. Additionally, morphologic differences were seen between passages. (B): Quantification of cell attachment revealed significant differences between media. (C): Colony-forming potential of the different media. It can be seen that StemPro significantly underperformed compared with the other media. The results are presented as mean ± SEM. Cell number was normalized to the cell number of each individual donor for A-MEM^{hPL10} at passage 1. †, Statistically different from all other media at $p < .05$. *, StemPro statistically different from all other media. $p < .05$. #, StemPro statistically different from all other media except A-MEM^{FCS} at $p < .05$. Scale bar = 500 μm. Abbreviations: A-MEM, α-minimum essential medium; CFU, colony-forming unit; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; hPL5, 5% human platelet lysate; hPL10, 10% human platelet lysate; P, passage; SVF, stromal vascular fraction.

pattern was observed for cells from all donors; however, the relative size of the CD146⁺ or CD146⁻ populations varied between donors. Finally, it did not appear that culture in media supplemented with either hPL or FCS promoted a particular immunophenotypic profile, upon qualitatively assessing the populations. Neither was any difference between A-MEM and DMEM observed (supplemental online Fig. 4).

DISCUSSION

When choosing media for culture of ASCs, selection is often based on a set of supportive characteristics as expansion, morphology,

and multilineage differentiation. Traditionally, ASCs have been cultured in DMEM supplemented with FCS, which has been found to support establishment and maintenance of ASCs while preserving their stem cell characteristics [1]. However, when expanding cells for subsequent clinical applications, regulatory perspectives should also be taken into consideration [52]. For this purpose, expansion in chemically defined media is appealing, as it ensures both a xenogeneic-free and a highly reproducible environment. We found, however, that StemPro, the chemically defined and xenogeneic-free medium used in this study, did not support the establishment of a viable culture of ASCs from SVF, although it was previously shown to satisfactorily support ASC expansion

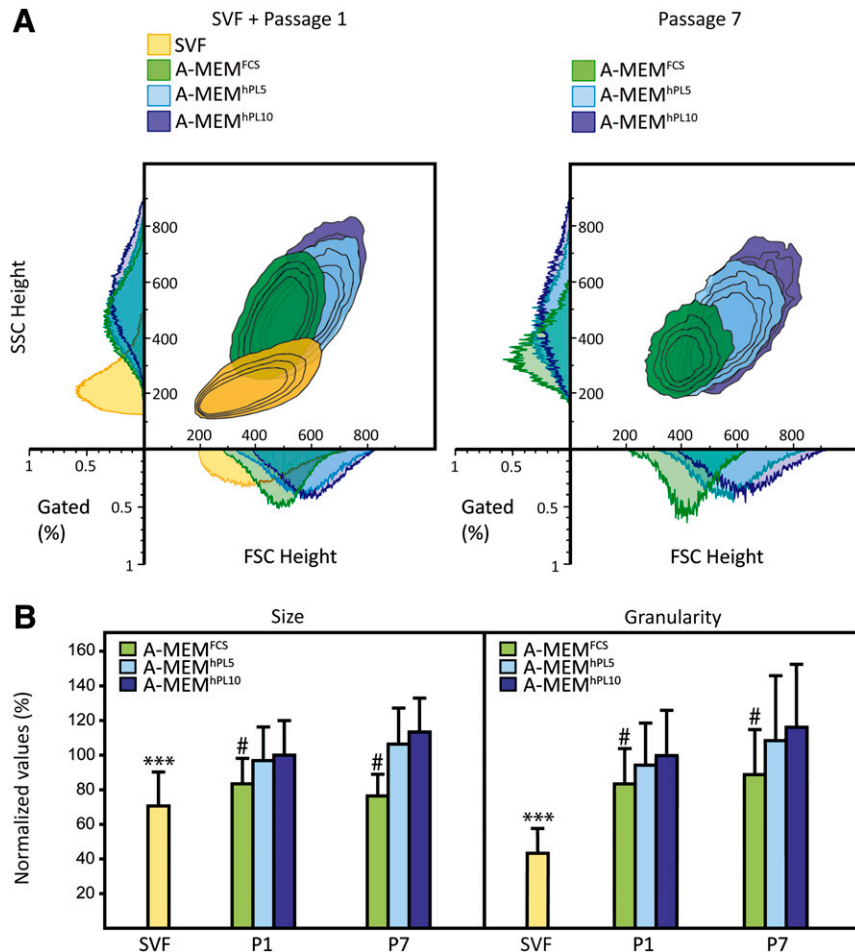


Figure 3. Size and granularity of adipose-derived stem cells (ASCs) cultured in different medium compositions. **(A):** Representative example from donor 1 showing the changes in size and granularity between the stromal vascular fraction, passage 1, and passage 7. Size was measured by mean of forward scatter height, and granularity was measured by mean of side scatter height using flow cytometry. The number of cells is presented as the percentage of total number of gated cells. **(B):** Average size and granularity for all donors reveal an increase in both size and granulation by first passage. The results are presented as mean \pm SD. Cell size and granularity were normalized to the cell size and granularity of each individual donor for A-MEM^{hPL10} at passage 1. ***, Statistically different from all other groups at $p < .001$. #, A-MEM^{FCS} statistically different from all hPL-supplemented media at $p < .001$. Abbreviations: A-MEM, α -minimum essential medium; FCS, fetal calf serum; FSC, forward scatter; hPL5, 5% human platelet lysate; hPL10, 10% human platelet lysate; P, passage; SSC side scatter; SVF, stromal vascular fraction.

[27]. This effect has also been described in previous studies [53]. The failure of StemPro to support establishment of ASC cultures could not be explained by inhibition of cell attachment to growth surface, as this medium was found to support attachment the most, probably supported by the CELLstart coating used for this medium as recommended by the manufacturer. However, in spite of supporting attachment of cells, StemPro failed to support both cell proliferation and generation of CFUs.

From a clinical viewpoint, the choice of a basal medium supplemented with hPL is another attractive option, to avoid the issues associated with the use of FCS. In this study, we compared A-MEM supplemented with FCS versus A-MEM supplemented with either 5% or 10% hPL. Regardless of the concentration, hPL supported ASC proliferation significantly better than FCS, which is in agreement with observations by others [38, 44, 54, 55]. The increase in cell yield did not appear to stem from higher numbers of cells attaching after seeding, nor from better yield of CFUs. However, larger colonies were observed in all hPL-supplemented medium compositions, which probably was a result of the faster proliferation in these

compositions. Additionally, ASCs cultured in hPL-supplemented media were larger and more granular than ASCs cultured in FCS-supplemented counterparts. Others have reported the opposite effects of hPL on ASCs' morphologic parameters, but using DMEM as basal medium [38, 55]. This shows the profound impact that a particular medium formulation has on the morphologic characteristics of ASCs. It has been suggested that morphologic properties may be linked to defined subpopulations within MSC cultures that display defined biological properties [56].

In agreement with earlier studies and the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy criteria for ASCs [2, 20, 57–59], >80% of the cells expressed CD73, CD90, and CD105 with passaging. As already mentioned, the limited amount of cells resulting from the SVF preparations restricted the number of conditions that could be assessed in parallel for each donor. Therefore, as StemPro has been proven unsuitable for initiation of ASC cultures, and DMEM supports ASC proliferation to a lesser degree than A-MEM, we chose to focus the analysis of surface

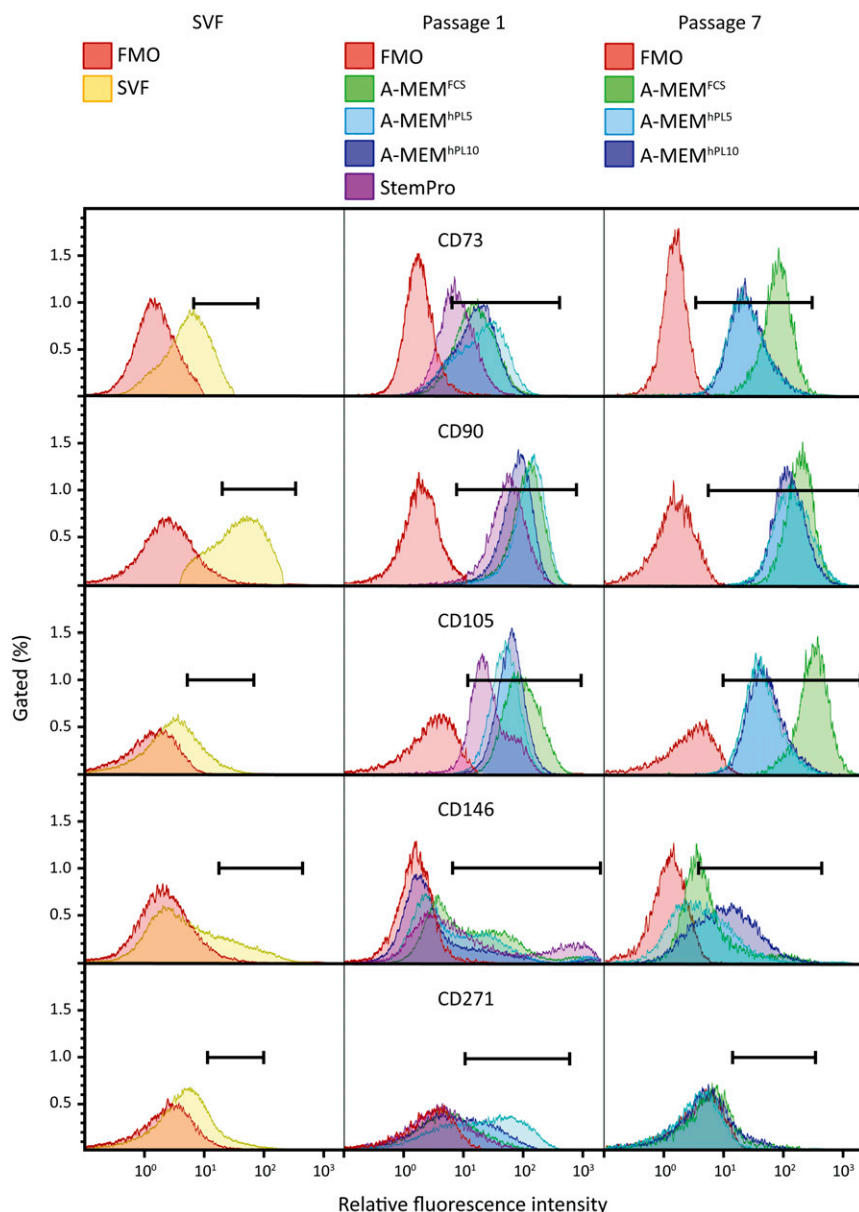


Figure 4. Flow cytometric profiles of surface markers obtained from SVF and adipose-derived stem cells (ASCs). ASCs were cultured in different medium compositions for one or seven passages. Differences between passages and medium compositions were found using flow cytometric analysis. Representative data are shown for donor 1. Abbreviations: A-MEM, α -minimum essential medium; FCS, fetal calf serum; FMO, fluorescence minus one; hPL5, 5% human platelet lysate; hPL10, 10% human platelet lysate; SVF, stromal vascular fraction.

markers on the effect of hPL and FCS supplementation of A-MEM. In addition to the presence of selected surface markers, the lack of expression of surface antigens CD31 and CD45 should be demonstrated. This lack of expression of CD31 and CD45 by ASCs cultured in conditions described in this article has been conclusively demonstrated by us and others in previous studies [27, 38, 46, 48, 53, 60, 61]. Thus, because of limitations on the number of different surface markers that could be simultaneously detected by flow cytometry, we chose not to perform an analysis of expression of CD31 and CD45.

Interestingly, when quantifying surface marker expression after seven passages, ASCs cultured in FCS were more bright in the expression of CD73 and CD105 and dim in CD146 compared with ASCs cultured in hPL. Also, continuous culture in either FCS or

hPL affected the distribution of ASCs within the subcategories of CD146⁺ and/or CD271⁺ cells. Whether and how this altered expression of the classic stem cell markers CD73 and CD105 reflects on the functionality of the ASCs is still uncertain, but CD73 has been suggested to be involved in differentiation [62] and regulation of the immune modulatory pathway together with CD105 [63, 64].

We also chose to investigate the effect of culture media on the expression of CD146 and CD271, which are not routinely included in ASC characterization. A high expression level of CD146 has been correlated with multipotency and possible a microvascular pericyte phenotype [65, 66]. Additionally, these cells have been shown to be more angiogenic than their CD146-negative counterparts, making them very attractive for regenerative

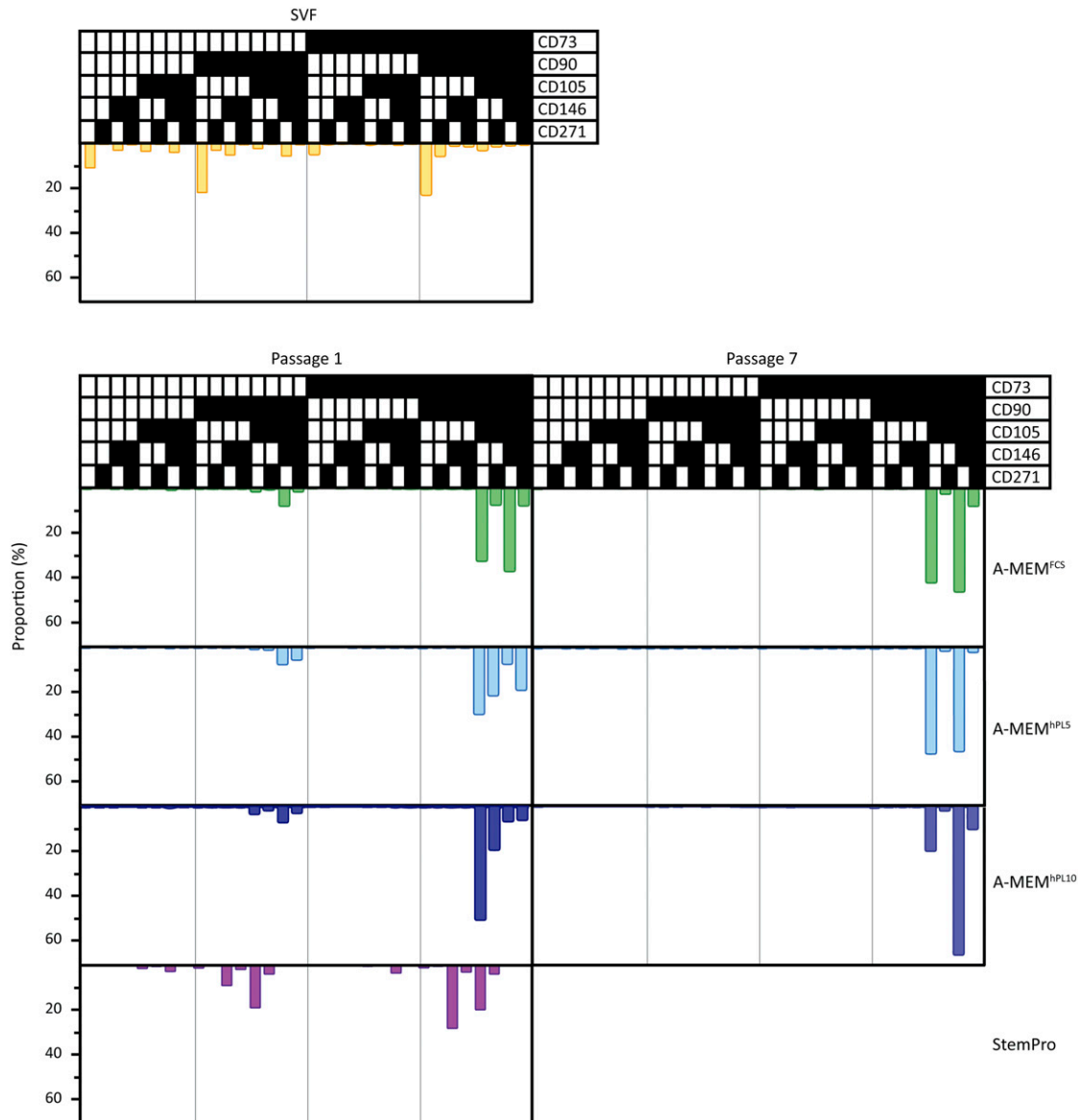


Figure 5. Flow cytometric multiparametric analysis of adipose-derived stem cell (ASC) subpopulations for each medium composition. By the first passage, ASCs cultured in all medium compositions became more homogeneous. Homogeneity was greater by passage 7. Overall, subpopulations had the same characteristics, but differences were found in the size of the different subpopulations. Representative data are shown for donor 1. ■, the marker was expressed; □, the marker was not expressed. Abbreviations: A-MEM, α -minimum essential medium; FCS, fetal calf serum; FMO, fluorescence minus one; hPL5, 5% human platelet lysate; hPL10, 10% human platelet lysate; SVF, stromal vascular fraction.

therapy [67]. Compared with SVF, there is a higher proportion of CD146⁺ cells in the ASCs. This proportion of approximately 40% positive and very positive cells remained relatively constant from passage 1 to passage 7. However, although the amount of highly expressing cells declined for all media, hPL preserved relatively higher expression levels than FCS, which may have implications for use of ASCs for treatment of ischemic diseases. The marker CD271 has been suggested to be a subpopulation of resting primitive MSCs [68]. In agreement with earlier studies by others [68], we found that the proportion of CD271⁺ cells decreased with passaging, and we did not observe differential effects of hPL and FCS.

CONCLUSION

In this work, we compared different types of media on the establishment and maintenance of ASC cultures. It was found that the chemically defined medium StemPro was unsuitable for initiation of ASC cultures from SVF. In addition, culture in hPL favored a high proliferation rate, and both FCS and hPL were appropriate for maintaining stem cell characteristics. However, culture in either FCS or hPL favored specific subpopulations within the ASCs in terms of the intensity of specific surface antigen expression. Apart from the classic markers for ASCs, we identified a number of subpopulations within the ASCs that mainly differed by their expression of CD146 and CD271. Thus, an important biologic measure to

take into account when choosing culture system and medium composition, besides the classic measures of proliferation and stem cell characteristics, might be the resulting composition of subpopulations, as these are affected by culture system and might be linked to regenerative potential. Further investigation of the regenerative potential of the different subpopulations is needed to reach more definite conclusions.

ACKNOWLEDGMENTS

We acknowledge provision of adipose tissue samples by the Teres Private Hospital and the technical assistance of O. Jensen. The work was supported in part by funds from Lily Benthine Lunds Fond (SR Grant 5652) and the Obelske Family Foundation (TF Grant 20114-18036). The funding sources had no influence on

study design, collection, analysis, interpretation of data, writing of the report, or the decision to submit the paper for publication.

AUTHOR CONTRIBUTIONS

S.R.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; F.M.N.: collection and assembly of data, data analysis and interpretation; C.P.P.: manuscript writing, final approval of manuscript; V.Z.: data analysis and interpretation, final approval of manuscript; T.F.: conception and design, manuscript writing, final approval of manuscript, financial support.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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