Human Platelet Lysates Promote the Differentiation Potential of Adipose-Derived Adult Stem Cell Cultures

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Abstract: Adipose tissue from liposuction is a rich source for human mesenchymal stem cells. This type of adult stem cell is ethically acceptable, that paved the way for research on their potential use in regenerative medicine. However, any clinical application of adult stem cells is impeded by the use of FBS as an animal-derived growth supplement. In addition, stem cell cultures gained importance as innovative human-based alternative to animal testing, *in vitro* toxicology, drug testing and safety assessment. Thus, animal-derived component-free culture protocols are mandatory for a successful application of human stem cell-based testing systems under humanized conditions.

Recently, we succeeded in using human platelet lysates (PL) as a serum alternative in the cell culture of a number of human and animal cell lines, and human mesenchymal stem cells. PL were prepared as cell-free extracts from activated donor thrombocytes.

The minimal criteria defining multipotent mesenchymal cells are (1) the capacity to adhere to plastic, (2) the expression of specific surface antigens (e.g. CD73, CD90, CD105) for undifferentiated state, and (3) the potential of the cells to differentiate into the adipogenic, chondrogenic and osteogenic lineage. In the present study, adipose-derived stem cells (ADSC) were used as cell model. ADSC were maintained under PL or FBS and then switched to the respective media to induce mesodermal differentiation. Differentiation endpoints were assessed by phase-contrast microscopy and by histochemical staining: (1) lipid droplets in adipocytes were stained by Oil red O, (2) proteoglycans in chondrogenic spheroids were detected by toluidineblue, and fine structure of spheroids was monitored by scanning electron microscopy, and (3) calcium deposits in differentiated osteoblasts were stained with silver nitrate (von Kossa staining). Adipogenic differentiation was further confirmed by quantitative real-time PCR of selected marker genes (PREF1 *vs*. FABP4). There were no differences between FBS- and PL-grown ADSC, indicative for retention of the differentiation potential of ADSC under animal-derived component-free culture conditions in PL-supplemented culture media. The degree of adipogenic and osteogenic differentiation was even more pronounced under PL compared to FBS.

Keywords: Human adult stem cells, platelet lysates, multipotent stem cell differentiation, mesodermal lineage.

INTRODUCTION

Adult mesenchymal stem cells reside in almost all tissues, denoted as stem cell niches [1-3]. Human mesenchymal stem cells are, in contrast to human embryonic stem cells, ethically harmless, that opened the research for their potential use in regenerative medicine [4]. A specific feature of stem cells is their potential of *self-renewal* in order to replenish the stem cell pool inside the niche. *Asymmetric cell division* enables one of the daughter cells to differentiate into specific cell types, while the sibling cell remains in undifferentiated state [5, 6]. Although present in rather small cell densities, human adult stem cells can readily be isolated from selected tissues e.g. cord blood, bone marrow [7] or adipose tissue [8]. The latter, obtained by liposuction, is a rich source for mesenchymal stem cells, called adipose-derived stem cells (ADSC) [9-11].

A number of criteria have been defined, that characterize human mesenchymal stem cells in culture [12, 13]. (**a**) Adherence of the cells to tissue culture treated polystyrene surfaces, where cells exhibit a fibroblast-like phenotype, (**b**) expression of specific surface markers, like CD73, CD90 or CD105, and (**c**) the potential of the cells to differentiate towards mesodermal lineages e.g. the adipogenic, chondrogenic or osteogenic lineage.

For more than 60 years, fetal bovine serum (FBS) has served as a culture medium supplement, providing *mitogenic* factors, hormones, growth factors, and cytokines that are essential for growth and proliferation of cultured cells [14]. Thus, FBS is still the gold standard as culture media additive. However, the use of FBS has been questioned in recent years for a number of reasons: (1) serum in general is an ill-defined mixture of components and may contain adverse factors like mycoplasma, viruses or prion proteins; (2) for somatic cells – except blood leukocytes – serum and serum proteins, respectively, are no physiological environment; (3) there are strong animal welfare concerns about the harvest of fetal blood; and (4) recent concerns about the origin, quality and purity of FBS [15-18]. In addition, any clinical use of cultured

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human cells is hampered by the fact that most cells have been expanded in FBS-containing media [19, 20]. Therefore, to ensure clinical grade cell therapy material, any type of cell has to be expanded under animal-derived component-free culture conditions. The options are manifold: serum-free, chemically defined culture media [14] or the use of human-based alternatives to FBS [21-26].

In a number of studies it was recently shown that human thrombocyte extracts, containing platelet-derived factors, can serve as a valuable alternative to FBS in cell culture media [19, 27- 36]. It is a well known fact, that it is serum and not plasma, that supports growth and proliferation of cells in tissue culture [37, 38], although human adult serum is not very effective due to the low content of growth promoting factors. Human thrombocytes are known to play an essential role in blood coagulation and to participate actively in wound healing processes by releasing a broad spectrum of growth factors, chemokines and signaling molecules through exocytosis of platelet α-granules [39-42]. Recently, we were able to show that α-granule releasates (platelet lysates, PL) can successfully replace FBS in culture media for a variety of human and mammalian cell lines [24] and human mesenchymal stem cells [25].

When mesenchymal stem cells are cultured under conditions other than high FBS supplementation, it is unequivocally important that the cells retain their specific features. For adult stem cells these attributes include self-renewal and multipotency i.e. growth and proliferation in PL-supplemented media by simultaneous retention of the undifferentiated state, and the capacity to differentiate into specific tissue lineages. In a preceding paper [25] we showed that ADSC can be grown in “humanized” culture media supplemented with PL thereby maintaining their undifferentiated state. A third question that has to be asked when innovative culture system are applied to ADSC is, whether the cells still can be triggered to differentiate into tissue-specific lineages [31, 43-45].

Here we present a comprehensive analysis of the mesodermal differentiation potential of FBS- and PL-grown ADSC. Differentiation of ADSC into specific tissue lineages was assessed by phase-contrast microscopy and histochemistryof adipocytes, chondrocytes and osteoblasts, respectively, and scanning electron microscopy of chondrogenic aggregates. Furthermore, selected marker genes for differentiated adipocytes were analyzed by quantitative real-time PCR.

MATERIALS AND METHODS

Platelet Lysate Preparation

The preparation of human platelet lysates (PL) was performed as described [24, 25]. As source material, expired human donor thrombocytes were used, that were provided immediately at the end of the shelf life (i.e. 5 days after donation). In brief, human thrombocyte concentrates, obtained by apheresis at the blood bank of the Innsbruck Medical University Hospital, were transferred under sterile conditions into 250 ml centrifugation cups and centrifuged at 6,000 $\times$ g for 20 min to remove platelet additive solution (PAS) and donor serum. The supernatant PAS was aspirated and the pellets were washed with 0.9% NaCl. Platelets were resuspended in 15 ml 0.9% NaCl with a final cell count of ~1.5 $\times$ 10^{10} platelets/ml. The suspension was stored at –20° C. Platelet lysates were prepared by three freeze/thawing cycles (freezing in liquid nitrogen and subsequent re-thawing). Six to eight platelet preparations were pooled and aliquots were stored at –20° C for up to 4 weeks. With this adjustment, 5% (v/v) PL were equivalent to 10% (v/v) FBS in terms of growth promoting capacity [24]. Before addition to serum-free basal culture mediain a final concentration of 5% (v/v), PL aliquots were thawed and spun at 8,000 $\times$ g for 10 min and the supernatants were taken.

Cell Culture

Commercially available adipose-derived human stem cells (ADSC), were used in the present study. ADSC, isolated from liposuction material [9], were purchased from Lonza Walkersville, Inc. (Cat. No. PT-5006) (www.lonza.com). Master stocks were frozen in liquid nitrogen and cells were used from passages 2 to 8.

Cultures were incubated at 37°C in a 5% CO_2 and 95% air atmosphere. Routinely, cultures were fed three times a week. Cell culture media were a 1:1 (v/v) mixture of DMEM (Dulbecco’s Modified Eagle’s Medium) base (Cat. No. D5030, Sigma-Aldrich), with 5.5 mM D-glucose, 2 mM L-glutamine, 26.2 mM $NaHCO_3$, and HAM´s F-12 nutrient mixture (Cat. No. N6760, Sigma-Aldrich), supplemented with 50µg/ml gentamicin (Cat. No. 15750-037, GIBCO), 2.5µg/ml amphotericin B (Cat. No. A2411, Sigma-Aldrich) and 10% (v/v) FBS(Biochrom/Berlin, Germany) or 5% (v/v) PL. Monolayers showing 80% of confluency were subcultured using 0.25% TrypLE™ (GIBCO) and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free buffered saline.

TrypLE™ is a novel recombinant trypsin preparation for serum-free cell culture that does not require inactivation by serum and/or protease inhibitors. Tissue culture plasticware and culture dishes were from Greiner (Greiner Bio-One, Kremsmünster, Austria), Sarstedt (Sarstedt, Inc., Newton, NC) and Becton Dickinson (Becton Dickinson Labware, Two Oak Park, Bedford, MA).

Differentiation Protocols

Differentiation of ADSC into mesodermal tissue lineageswas performed in parallel series on ADSC cultured in media with either 10% FBS or 5% PL. ADSC were grown for 7 days and then transferred to the respective differentiation media, containing selected inducers of differentiation [45, 46] (see below).

Adipogenic differentiation was induced with StemPro® Adipogenesis Differentiation Kit (GIBCO Cat. No. A10070-01), containing dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, and indomethacin. Cultures were maintained for7 days with changes in induction medium every 48 h. After 1 week, differentiated cells were fixed with paraformaldehyde (10% v/v in phosphate-buffered saline, PBS), rinsed in PBS and incubated for 10 minutes with Oil Red-O to stain intracellular lipid droplets. For gene expression analysis adipocyte cultures were harvested at day 0, day 5 and day 14 of adipogenic induction (see below).

For osteogenic differentiation, StemPro® Osteogenesis Differentiation Kit (GIBCO Cat. No. A10072-01) was applied, which is supplemented with dexamethasone, β-glycerphosphate, and ascorbic acid. Medium was changed 3 times a week. After 3 weeks, cells were fixed with 10% paraformaldehyde and calcium deposits were stained with von Kossa silver nitrate staining.

For chondrogenic differentiation, cells were pelleted and cultured in 15 ml conical Falcon tubes [47]. Cell aggregates were maintained in chondrogenic induction medium (StemPro® Chondrogenesis Differentiation Kit, GIBCO Cat. No. A10071-01) containing bone morphogenetic protein-6 (BMP-6), recombinant human transforming growth factor-β1 (TGF-β1), ascorbate-2-phosphate, dexamethasone, and sodium pyruvate. Induction medium was replaced 3 times a week. After 2 weeks in culture, tissue spheres were collected, fixed in 4% paraformaldehyde in PBS, dehydrated over a series of ethanol, and embedded in paraffin for histological microtomy. Semi-thin sections were stained for proteoglycans with 0.25% (w/v in distilled water) aqueous toluidine blue. In parallel, chondrogenic aggregates were fixed, dehydrated, critical point dried, and prepared for scanning electron microscopy (SEM).

Quantitative Real-Time PCR

For quantitative real-time PCR (qRT-PCR), total RNA was isolated by phenol-chloroform extraction (QIAzol®, QIAGEN, Cat. No. 79306) from confluent ADSC control cultures, and from cultures after 5 and 14 days in adipogenic differentiation media, respectively (Figure **1b**). The isolated RNA was precipitated by isopropanol. After centrifugation at 12.000 ×g, the supernatant was poured off, and the pellet was washed with 75% ethanol and centrifuged again. The final pellet was dried and dissolved in RNase–free water. First-strand cDNA was synthesized from 50ng RNA using MuLV reverse transcriptase (N808-0018, Applied Biosystems), dNTP Mix (R0192, Thermo Scientific) and Random Hexamer Primer (SO142, Thermo Scientific).

PCR reagents included a Taqman Gene Expression Master Mix (4369016; Applied Biosystems) and predesigned primers for PREF1 (preadipocyte factor-1) (Hs00171584_m1, Life Technologies) and FABP4 (fatty acid-binding protein 4) (Hs01086177_m1, Life Technologies) were used to determine gene expression levels.18S rRNA (4310893E, Life technologies) was used as an internal reference to normalize the target genes by the $2^{-\Delta\Delta CT}$ method.

qRT-PCR was performed on a Applied Biosystems® 7500 Fast Real-Time PCR System. PCR was started at 50°C for 2 min and then 95°C for 10 min to activate polymerase, followed by 40 cycles at 95°C for 15 s and at 60° C for 1 min.

Statistical analysis was performed using the unpaired Student's *t*-test.

RESULTS

The Differentiation Potential of ADSC is Retained under PL-Supplemented Growth Conditions

A prerequisite for a successful substitution of FBS by PL in human stem cell cultures is the full retention of adult stem cell attributes under the applied culture conditions. We recently showed that substitution of FBS by PL in culture media for mesenchymal ADSC supported attachment, growth and proliferation of the cells, comparable or even superior to cultures supplemented with 10% FBS. Furthermore, ADSC

retained their undifferentiated phenotype as assessed by the expression of selected surface markers: CD73, CD90 and CD105. CD45 served as negative control [25]. For multipotent mesenchymal stem cells, additional attributes include the potential to differentiate into mesodermal lineages, the adipogenic, chondrogenic and osteogenic lineage [43-45].

To this end, ADSC were cultured to subconfluence in either FBS-supplemented or PL-supplemented media for 7 days. Thereafter, parallel cultures were switched to specific differentiation media. The differentiation endpoints were assessed by phase-contrast microscopy of differentiated cell layers (Figure **1**), by histochemical staining of cells (Figure **2**), and scanning electron microscopy (Figure **3**). In addition, expression of selected marker genes was proven by qRT-PCR (Figure **4**).

For induction of adipogenesis cultures were incubated for 7 days or longer, until differentiated adipocytes were visible by phase contrast microscopy. A clear shift from the characteristic fibroblast-like morphology of ADSC control cultures (Figure **1a**) to a more rounded shape of differentiated adipocytes with distinct lipid vacuoles in the cytoplasm could be detected (Figure **1b**). Lipid droplets accumulating in differentiated adipocytes were stained by Oil Red-O stain (Figure **2a**). Furthermore, selected adipocyte differentiation markers were determined by qRT-PCR. Preadipocyte factor-1 (PREF1) is specifically expressed in preadipocytes, but is extinguished during adipocyte differentiation. Thus, PREF1 serves as marker for preadipocytes [48]. Fatty acid-binding protein FABP4 belongs to a set of genes that are highly expressed in mature adipocytes [49]. As shown in Figure **4**, PREF1 mRNA gradually decreased, while FABP4mRNA levels strongly increased after 14 days of adipogenic differentiation.

The chondrogenic differentiation protocol is designed to induce chondrogenic aggregates in 3D micromass cultures for 14 days [47], in which proteoglycan structures can be stained in histological sections of the aggregates with toluidine blue (Figures **2b**, **3a**). The development of proteoglycans in intact chondrogenic aggregates was confirmed by scanning electron microscopy of the sub-microscopical fine structure (Figure **3b**). In parallel, PL-grown ADSC cultures were continued in chondrogenic differentiation medium in monolayer culture. As depicted in Figure **1c**, ADSC dramatically changed their phenotype from a fibroblast-like appearance to epithelial-like cells with the accumulation of extracellular matrix material.

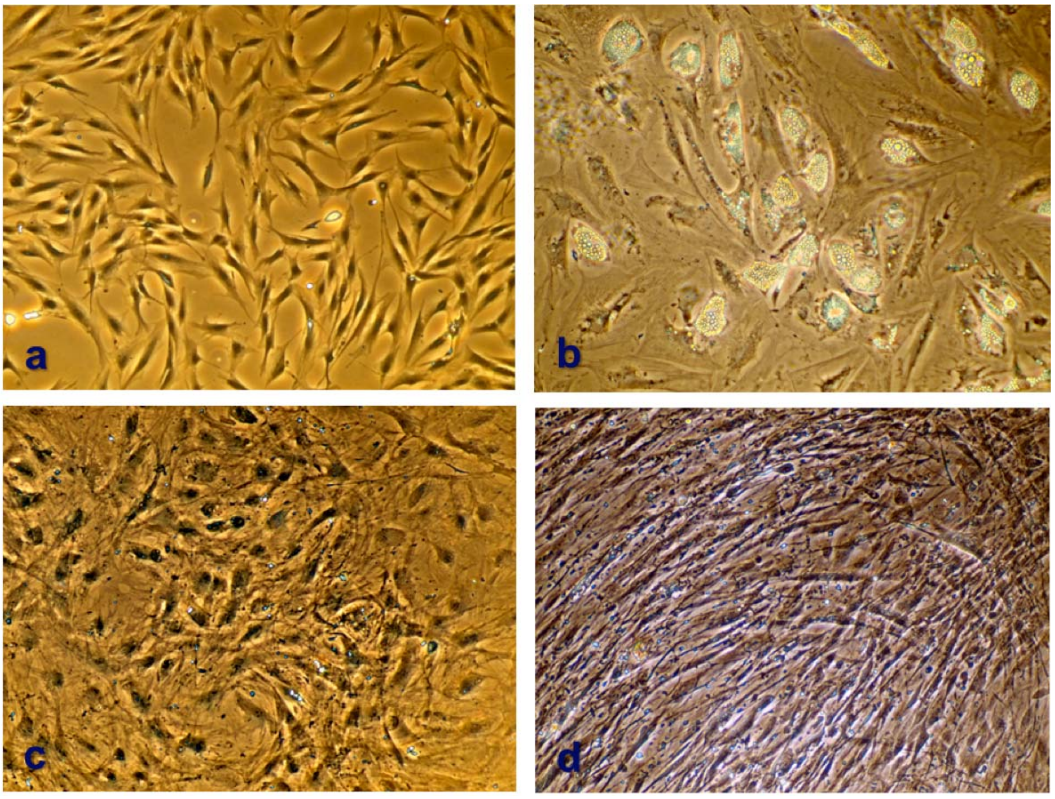

Figure 1: Phase-contrast micrographs of ADSC in control medium supplemented with PL (**a**), and differentiated monolayer cultures of adipocytes (**b**), chondrocytes (**c**), and osteoblasts (**d**). Magnification **a**, **c**, **d**100 ×; **b** 250 ×.

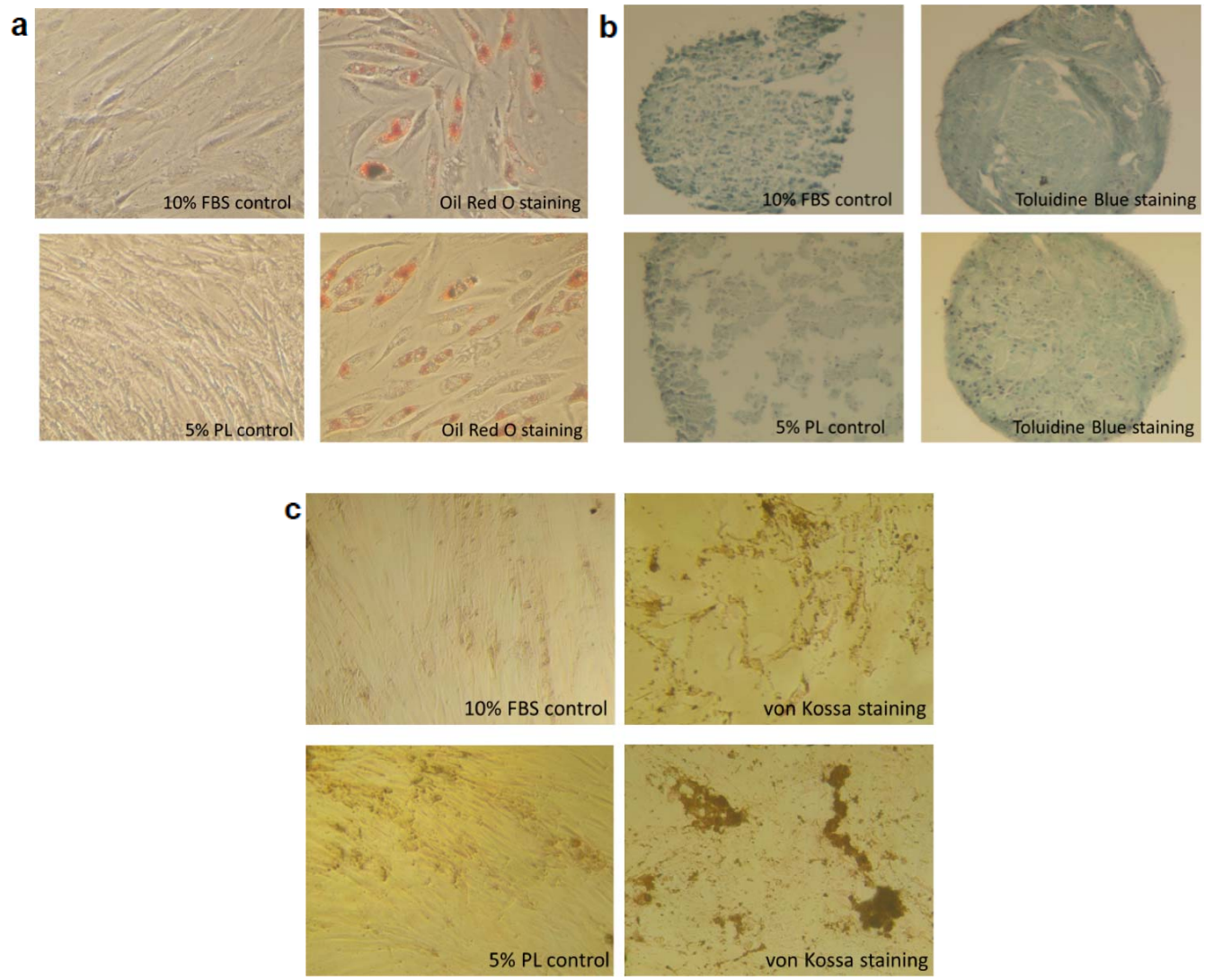

Figure 2: a: Histochemical staining (Oil Red-O) of differentiated adipocytes and comparison of FBS-grown (upper panels) and PL-supplemented ADSC cultures (lower panels).

b: Histochemical staining (toluidine blue) of semi-thin sectioned differentiated chondrocyte aggregates and comparison of FBS-grown (top panels) and PL-supplemented ADSC (bottom panels).

c: von Kossa-staining of calcium deposits in differentiated osteoblasts and comparison of FBS-grown (upper panels) and PL-supplemented ADSC cultures (lower panels).

Also, the differentiation of ADSC into osteoblasts was monitored at the light microscopical and histochemical level, shown in Figure **1d** and **2c**. PL-grown ADSC were maintained in osteogenic differentiation medium for 21 days. The phenotypic changes into osteoblasts are well pronounced (Figure **1d**), which is further confirmed by von Kossa staining of calcium deposits in differentiated osteoblasts.

It must be emphasized, that the trilineage differentiation protocols were run in parallel with FBS-grown and PL-grown ADSC cultures, respectively. As can be seen in Figures **2** and **3**, no differences between the two culture conditions could be detected. Indeed, adipogenic and osteogenic differentiation of ADSC was even more pronounced with cells cultured in PL-supplemented media, compared with FBS-grown cells.

DISCUSSION

Research on human mesenchymal stromal/stem cells (MSC) gained enormous attention, since on the one hand, the cells are ethically acceptable and can easily be isolated from a variety of human tissues, and on the other hand, MSC retained their stem cell plasticity i.e. the capability to differentiate into multiple mesodermal cell types [44, 50-52]. ADSC are easy

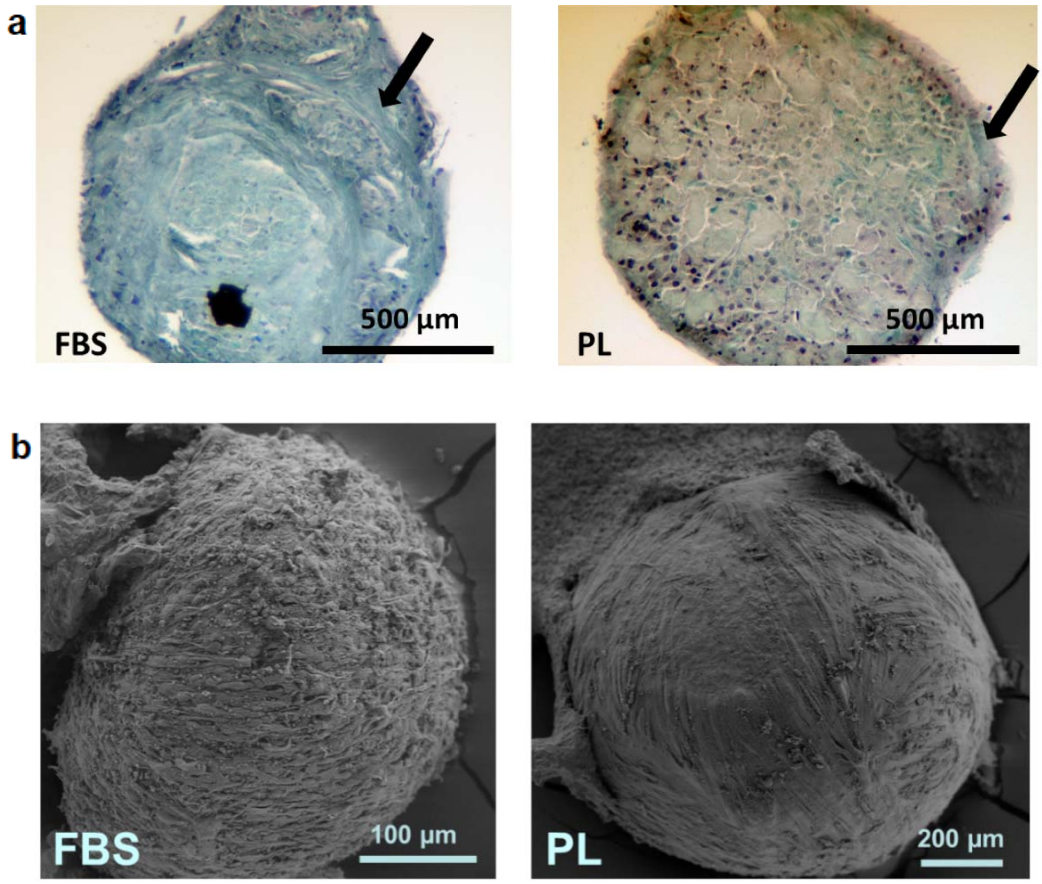

Figure 3: a: Semi-thin sections of chondrogenic aggregates, differentiated from FBS-grown (left) and PL-supplemented ADSC (right), with toluidine blue-stained proteoglycans (arrows).

b: Scanning electron micrographs of chondrogenic aggregates, differentiated from FBS-grown (left) and PL-supplemented ADSC (right). Proteoglycan extracellular matrix structures are shown at high magnification.

accessible mesenchymal cells after liposuction surgery, and have become popular adult stem cells in the emerging field of regenerative medicine [9, 53].

After having shown that PL are a valuable surrogate for FBS in ADSC cultures [25], the aim of the present study was to expand our work on the differentiation of ADSC. Here we show, that ADSC retain their full differentiation potential under PL growth conditions and that the cells can be triggered to differentiate into the adipogenic, osteogenic and chondrogenic lineages.

Trilineage differentiation was induced using established protocols of differentiation media. The compositions of the various differentiation kits have been reviewed in detail [45]. Basal growth media are supplemented with selected agents to induce lineage-specific differentiation. The complex media differ in their quantitative composition; however, qualitatively the supplementation of media with specific inducers is fairly uniform. Adipogenic inducers are dexamethasone, IBMX, insulin and indomethacin. Chondrogenic differentiation media contain dexamethasone, ascorbate-2-phosphate, BMP-6, TGF-β1, and sodium pyruvate, while osteogenic differentiation is achieved by dexamethasone, β-glycerophosphate, and ascorbic acid. Lineage-specific differentiation (Figure **1**) was verified at the histochemical level (Figure **2**). In addition, chondrogenesis was assessed at the ultrastructural level (Figure **3b**), and adipogenic differentiation was monitored at the molecular biological level (Figure **4**).

Adipogenic differentiation is a multistep process from preadipocytes to mature adipocytes (see below) [54-56]. During adipogenic differentiation, fibroblast-like ADSC convert to a spherical shape (Figure **1b**). Differentiated adipocytes were visualized by Oil Red-O staining of neutral lipid droplets, which accumulate in small vacuoles (Figure **2a**).

After osteogenic induction, ADSC underwent typical morphological changes from fibroblast-like cells to a

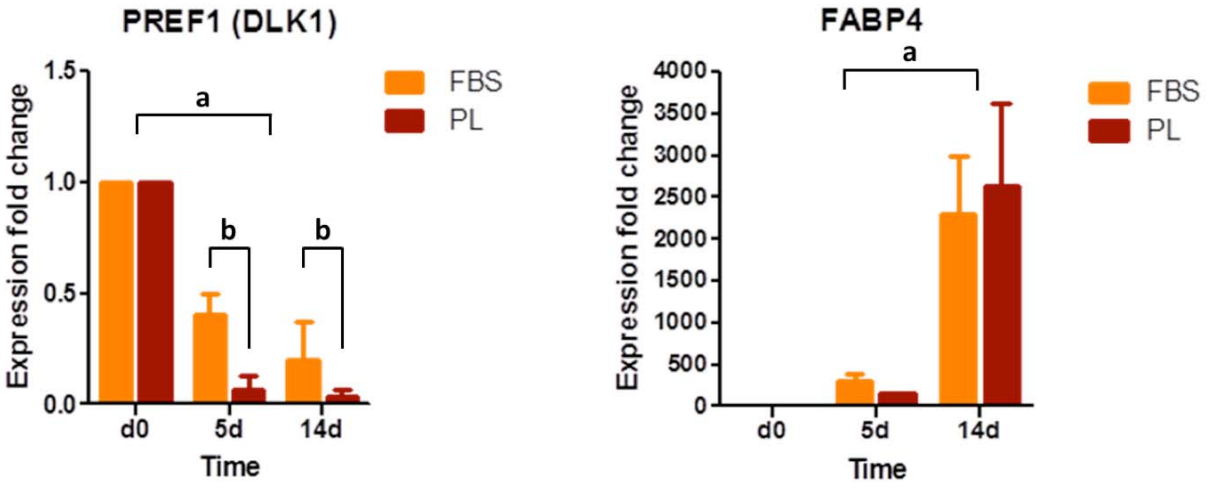

Figure 4: Changes in mRNA levels of PREF1, a preadipocyte marker (left panel), and of FABP4, a marker for mature adipocytes (right panel), during adipogenic differentiation of ADSC. Control cultures (d0) and ADSC after 5 and 14 days in adipogenic differentiation media, respectively, were harvested for mRNA isolation. Gene expression levels were determined by qRT-PCR. The baseline expression levels in control cultures (d0) were set to 1.0. a) The decreases in PREF1 mRNA as well as the increases in FABP4 mRNA levels are statistically significant from controls ($p<0.01$). b) In addition, the differences between FBS and PL are statistically significant within the time courses of PREF1 mRNA decrease ($p<0.01$).

spindle-shaped osteoblast phenotype, capable of mineralization (Figure **1d**). Calcium deposits which develop during osteogenic differentiation were confirmed by von Kossa staining (Figure **2c**).

Chondrogenesis was induced by forming cell aggregates grown in micromass cultures in chondrogenic differentiation medium. Chondrogenic differentiation can also be performed in monolayer cultures (Figure **1c**); however, the efficiency of chondrogenic aggregates is higher, since it mimics cartilage tissue *in vivo* [57]. Chondrogenic differentiation was verified by toluidine blue staining of proteoglycans in semi-thin sections of chondrogenic cell pellets (Figures **2b** and **3a**) as well as by scanning electron microscopy of chondrogenic aggregates, where specific proteoglycan structures could be observed at the ultrastructural level (Figure **3b**). Both methods approved the chondrogenic differentiation potential of FBS- and PL-treated ADSC.

As mentioned above, adipocytes derive from multipotent mesenchymal stem cells, like ADSC, in a multistep maturation process [55, 58]. However, at present, it is still difficult to describe distinct cellular intermediates during adipogenic maturation. When a stem cell is committed to the adipocyte lineage, called determination, a first step involves the conversion of a stem cell to a preadipocyte. In a second phase, known as terminal differentiation, the preadipocyte converts to a mature adipocyte (Figure **5**). Therefore, in the present study, we attempted to describe the cellular intermediates between ADSC and mature adipocytes at the molecular biological level.

To this end, the stages of adipocyte differentiation were examined by determination of the rates of expression of two candidate genes involved in adipocyte maturation (Figure **4**). PREF1 (DLK1) is a preadipocyte-specific marker and is absent in mature adipocytes [48, 59]. FABP4 (fatty acid-binding protein), a lipid transporter, is a pivotal marker of the mature adipocyte gene signature [49, 60, 61] (Figure **5**). PREF1 (preadipocyte factor-1, also called delta-like protein 1, DLK1) is specifically expressed at high rates in preadipocytes. It is a molecular gatekeeper of adipogenesis, which maintains the preadipocyte state, thereby preventing adipocyte differentiation [62]. PREF1 activates the ERK/MAPK pathway with down-stream upregulation of Sox9, which in turn suppresses the expression of two major factors for adipogenic induction, C/EBPβ and C/EBPδ,thereby inhibiting adipogenesis. In the adipogenic induction protocolPREF1 is down-regulated by dexamethasone, which is present in the differentiation medium cocktail, leading to the initialization of adipogenesis. The subsequent activation of PPARγ and C/EBPα promotes adipogenesis and results in the expression of FABP4 and other adipocyte-specific genes that play a key role in insulin sensitivity, lipogenesis and lipolysis [49].

As depicted in Figure **4**, the expression of PREF1 mRNA declined gradually over time by 60 – 80% in FBS-treated cells after 14 days in culture compared to

day 0, and even by 90% in PL-grown cultures. In contrast, FABP4 mRNA is upregulated2.500-fold in both, FBS- and PL-grown ADSC after 14 days of adipogenic differentiation. From the gene expression data it can be concluded, that the cells converted from a preadipocyte stage to the state of terminally differentiated adipocytes (see also Figures **1a** and **2a**).

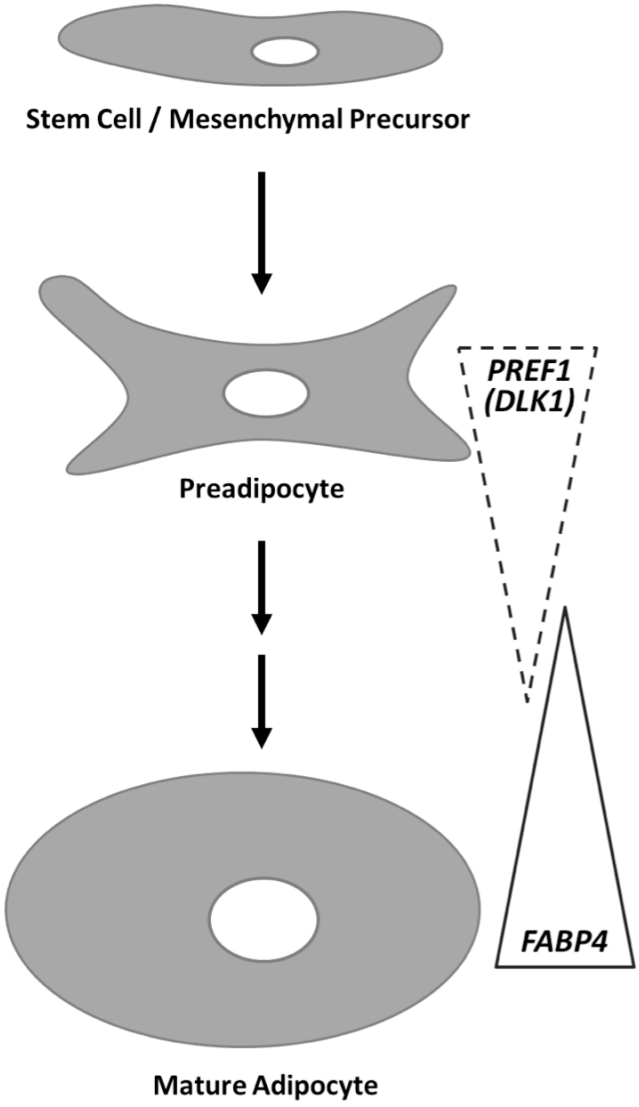

Figure 5: The stages of adipocyte differentiation. Mesenchymal stem cells/precursor cells can be triggered to differentiate into mature adipocytes. The transition from preadipocyte to mature adipocyte is characterized by changes in specific gene expression pattern. In the intermediate preadipocyte state, PREF1 (DLK1) is expressed, which gradually decreases during progression of adipocyte maturation (dashed triangle). The signature of gene expression in mature adipocytes includes – among other adipocyte genes – FABP4, which is highly expressed at this stage (solid triangle). For original data see Figure **4**.

The expression pattern of the osteogenic marker gene RUNX-2 was recently determined in PL-grown mesenchymal stem cells after osteogenic induction [51]. Also in this study, no significant differences in the differentiation potential of PL- *vs*. FBS-cultured cells could be observed.

In summary, PL are a proven alternative for FBS in ADSC cultures [25] and for culturing continuous human and animal cell lines [24]. ADSC cultured in the presence of PL adhere to plastic tissue culture vessels, proliferate, and remain in the undifferentiated state of multipotent mesenchymal stem cells [25]. A major task of the present study was to investigate whether animal-derived component-free medium supplements, like PL, are also supportive in the induction of ADSC differentiation. Here we were able to show that ADSC cultured under PL-supplemented conditions, retained their full potential to differentiate into mesodermal tissue lineages. The retention of the differentiation capacity of human mesenchymal stem cells under xeno-free culture conditions may become an important aspect in future cell replacement therapies. Thus, human platelet lysates may open new avenues in clinical stem cell applications [32].

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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