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Characterization of ocular surface epithelial and progenitor cell markers in human adipose stromal cells derived from lipoaspirates

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Running head: Human mesenchymal stem cells and the ocular surface

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Abstract:

Purpose. The goal of this study was to characterize and compare mesenchymal stem cells from adult human adipose tissue (ADS cells) with progenitor cell lines from the human corneoscleral limbus, and to analyze their potential for expression of epithelial markers.

Methods. Stem cell markers (CD34, CD90, p63 and ABCG2) and epithelial cell markers (CK3/76, CK12, CK76, CK19 and CK1/5/10/14) were analyzed by immunostaining, flow cytometry, Western blot and PCR methods. We assayed adhesion and proliferation on different extracellular matrix proteins.

Results. ADS cells expressed a set of progenitor cell markers, including p63 and ABCG2. CK12 expression in ADS cell cultures increased spontaneously and progressively by differential adhesion, which demonstrates the cells' potential and capability to acquire epithelial-like cell characteristics. We observed an increase in the adhesion and proliferation of ADS cells seeded onto different basement membrane extracellular matrix proteins. Laminin substrates reduced the proliferative state of ADS cells.

Conclusions. The expression of putative stem cell markers (CD90, ABCG2 and p63) and cytokeratins (CK12 and CK76) supports the hypothesis that ADS cells have self-renewal capacity and intrinsic plasticity that enables them to acquire some epithelial-like characteristics. Therefore, adult ADS cells could be a potential source for cell therapy in ocular surface regeneration.

Introduction

The corneal surface is covered with highly specialized epithelia that are derived from progenitor cells located in the basal layer of the corneoscleral limbus. The corneal epithelium regenerates rapidly and must be replaced to maintain the proper role of the cornea. This is an important requisite for ocular surface integrity and also preserves good refractive and visual function[1,2]. It is now known that limbal stem cells (LSC) form a multipotent progenitor cell pool that acts as a proliferative reservoir of self-renewing corneal epithelium and demonstrates many properties typical of an adult stem cell population[3,4].

Some putative limbal stem cell markers have been found, but none have been verified[4-8]. Despite the large number of proteins, the following potential markers for LSC have been proposed: changes in the cytokeratin (CK3 and CK12) pattern; elevated levels of the transcription factor p63 (ΔNp63α); several growth factor receptors, including both EGF and TGF-β receptors; differential expression of several integrins coupled with their underlying basement membrane proteins; and ATP-binding cassette transporter protein ABCG2 expression on the cell membrane[5-8]. This has led to a better understanding of how the corneoscleral limbus is involved in regenerating the corneal epithelium.

Insufficient or depleted LSC leads to the migration and growth of conjunctival elements on the corneal surface, which becomes a “conjunctivalization” event. The consequences are corneal opacification, new vessel formation and progressive, marked loss of transparency and vision[9]. Several pathological conditions that damage the eye surface, such as acid or alkali burns, Steven-Johnson syndrome, cicatricial pemphigoid of the eye and hereditary aniridia, may cause severe LSC deficiency and follow the above

pathophysiological steps[9,10]. Corneal transplants are currently the most effective treatment, with recovery of vision in many cases when the cornea has become opaque. However, keratoplasty will fail in corneal opacification due to ocular pathology that leads to some degree of LSC deficiency. With biotechnology improvements in identifying and characterizing stem cells, and advances in cell isolation and culture techniques for selecting and expanding *ex vivo*, regenerative medicine that employs cell therapy is now a promising approach for treating several pathologies that affect the ocular surface[9,10].

Human adult mesenchymal stem cells (MSCs) derived from bone marrow or adipose tissue have been shown to have multilineage potential. They have been applied experimentally in tissue-engineering applications or other cell-based therapies[11-13]. Human adipose tissue can be easily obtained from liposuction aspirates separated into fatty and fluid portions. Cells isolated from the fatty portion are termed *processed lipoaspirate* (PLA) cells and mainly contain adipose-derived stromal (ADS) cells[13,14]. Recently, a cell population with a significant number of progenitor cells was characterized from the fluid portion of liposuction aspirates and defined as *liposuction aspirate fluid* (LAF) cells[15].

In recent years, *in vitro* and *in vivo* approaches have shown that ADS cells have the potential plasticity to differentiate not only into adipogenic, chondrogenic, osteogenic, myogenic and cardiomyogenic lineages[14, 16-18], but also into neuronal, glial, endothelial and hepatic cell lines[19-22]. This indicates that ADS cells have excellent therapeutic applications. However, there is little research about the utility of these cells in regenerative medicine in the field of ocular pathology. In one study[23], human adult ADS cells were employed as a cell source for regenerating the corneal stroma, in which

keratocytes are the main cell type of mesenchymal origin. Diseased corneas have been repopulated and repaired in an animal model and transparency was preserved for several weeks after adipose-derived progenitor cell therapy[23].

Furthermore, recent studies[24-25] showed that adult epidermal stem cells derived from skin keratinocytes could be cloned and differentiated into both neural and mesodermal progenies. However, there is very little knowledge about the potential of MSCs to take on some molecular epithelial-like characteristics. On the basis of our results, we believe that human adult ADS cells obtained from lipoaspirates could also be a cell source for corneal LSC substitution to regenerate the ocular surface.

Material and methods

Cell cultures

Murine fibroblast 3T3 Swiss albino (3T3-SA, CCL-92) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Before use, confluent cells were incubated with 10 µg/ml mitomycin-C (MMC; Sigma-Aldrich) for 2 hours at 37°C in a humidified atmosphere containing 5% CO_2. After this, the fibroblasts were harvested and plated onto cell culture dishes at 1.5 x 10^4 cells/cm^2 for feeder-layer use.

Human corneal epithelial cells and limbal epithelial cells (LSC)

Cadaveric adult human limbal tissue from different donors was obtained from the Eye Bank of the Transplant Services Foundation (TSF; Barcelona, Spain) and the *Centro de Oftalmología Barraquer* (COB, Barcelona, Spain). Informed consent for the use of this human tissue for experimental purposes was obtained in accordance with the Declaration of Helsinki. Any active transmissible infections were ruled out by serological analyses. LSC was isolated according to previous protocols[26, 27]. Corneal epithelial cells were obtained by mechanical scrapping of central corneal epithelium, avoiding the perilimbic region.

Isolation and culture of processed lipoaspirate cells (ADS)

Human adipose tissue aspirates were collected from plastic liposuction procedures, according to the principles outlined in the Declaration of Helsinki. Informed consent was obtained from patients and active transmissible infections were ruled out by serological

analyses. MSCs from fresh human lipoaspirates were cultured as described elsewhere[13].

Differentiation experiments

Cells were plated at 10^4 cells/cm^2 and cultured in ADS medium (DMEM, 10% FBS, 2 mM L-glutamine, 10 mM Hepes and antibiotics) for 24 hours. Afterwards, the medium was changed to an induction medium[12-14], which was maintained for 2 or 4 weeks. The medium was changed every 48 hours.

Adipogenesis. This was carried out in an adipogenic induction medium[13,14] containing ADS medium supplemented with 0.5 mM isobutyl methylxanthine, 1 μM dexamethasone and 200 μM indomethacin. Experiments were performed at 15 and 28 days. Adipogenic differentiation was confirmed by Oil Red O staining.

Osteogenesis. Osteogenic induction was performed in ADS medium with 1 μM dexamethasone, 50 μg/ml ascorbic acid and 10 mM glycerol 2-phosphate[13,14]. Experiments were carried out at 18 and 21 days. Osteogenic differentiation was confirmed by Alizarin Red staining and alkaline phosphatase activity quantification in cells (Sensolyte® pNPP Alkaline Phosphate Assay Kit; AnasPec, Tebu-bio).

Flow cytometry analysis

Cells at 10^5 cells/ml were incubated with fluorescent conjugated antibodies for 30 minutes at room temperature (RT) in the darkness. FITC-conjugated anti-human CD34 (CD34, clone 581/CD34; Biosciences), FITC-conjugated anti-human CD90 (CD90, clone 5E10; BD Biosciences) and phycoerythrin-conjugated anti-human Bcrp1/ABCG2

(ABCG2, clone 5D3; R&D Systems) were used. Then, cells were fixed and stored at 4°C until they were analyzed in an FC 500 Argon Laser Cytometer (Beckman Coulter Inc., Fullerton, CA, USA). All data were analyzed by the Cytomation Summit program (Cytomation Summit Software, Version 3.1; Fort Collins, Colorado, USA).

Immunocytochemistry

Cells grown to semi-confluence on glass coverslips were fixed, permeabilized and blocked. Coverslips were then incubated with primary monoclonal antibodies (mAbs) for 60 minutes at 37°C in a humid chamber. An anti-cytokeratin 3/76 (CK3/76, clone AE5; Chemicon) or anti-cytokeratin 1/5/10/14 (CK1/5/10/14, clone 34βE14; Novocastra) mAbs was used. After several washes in PBS solution, an FITC-conjugated rabbit anti-mouse immunoglobulin (DakoCytomation) was added for 30 minutes at RT. Cell nuclei were stained with bis-benzimide (Hoechst 33342, Sigma-Aldrich). Finally, the coverslips were mounted upside down with mounting medium (DakoCytomation). Cells were observed in an Olympus BX61 epifluorescence microscope (Olympus R-FTL-T, Olympus America Inc. Center Valley, PA, USA), coupled with an Olympus DP Controller Program for digital image acquisition.

Electrophoresis and Western blotting

For the Western blot analysis, a total cell extract was dissolved in SDS-loading buffer. The resulting lysate was electrophoresed on 10% SDS polyacrylamide gel (SDS-PAGE). The separated proteins were transferred overnight at 35 volts to nitrocellulose transfer membranes (BD Bioscience). The membranes were blocked for 1 hour to avoid non-

specific binding sites. The primary mAbs anti-tubulin (Sigma), anti-cytokeratin 19 (CK19, clone 170.2.14; Boehringer Mannheim), mAbs anti-p63 (p63, clone 4A4; Chemicon), mAbs against CK1/5/10/14 (Novocastra), mAbs against CK3/76 (Chemicon), and pAbs anti-CK12 (H-60; Santa Cruz) were added to membranes and incubated for 3 hours at RT or overnight at 4°C. After several washes, horseradish peroxidase-conjugated goat anti-mouse or swine anti-rabbit immunoglobulin (DakoCytomation) was added for 90 minutes at RT. Protein bands were revealed using an enhanced chemiluminescence substrate (ECL, Biological Industries, Reactiva, Barcelona, Spain) and were recorded on an autoradiography film (Kodak™, Rochester, NY, USA).

Reverse-transcription (RT) and real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using the PureLink Micro-to-Midi total RNA Purification System Kit (Invitrogen). RNA samples were reverse transcribed using Super-Script III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Several primers were designed for ABCG2, p63 (ΔNp63α), cytokeratins 3 (KRT3), 12 (KRT12) and 76 (KRT76), using the Primer 3 Software on the web (Steve Rozen and Helen J. Skaletsky [2000]; http//primer3.sourceforge.net). qRT-PCR was carried out using a StepOne instrument (Applied Systems, Foster City, CA, USA) and reaction mixtures were composed of 200 nM of primers and iCycler SybrGreenER Supermix solution (Invitrogen). Triplicate samples were amplified (43 cycles: 95°C 15 sec, 59°C 15 sec, 72°C 30sec) for each marker. The dilution series (1-10-100) of the specific PCR product of interest was prepared to determine the standard curve by relative quantification. To control the specificity of the reaction, melting-curve analysis was performed after amplification. The

amplification of 18S rRNA (RRN18S; TATAA Biocenter, Biotools, Spain) was used as a normalization control.

Polymerase chain reaction (PCR) analysis

DNA (cDNA) obtained by reverse transcription of total RNA from ADS cells was used in the PCR experiments. Primers for cytokeratins 3 (KRT3) and 12 (KRT12) were applied at 200 nM with 0.5 units of *Taq* DNA Polymerase (Platinum® *Taq* DNA Polymerase, Invitrogen) in the presence of 50 nM $MgCl_2$. We performed 40 cycles (95°C 45 s, 59°C 10 s and 72°C 15 s) and products were electrophoresed in 2% agarose gel for 1 hour. Ethidium bromide was used to visualize the PCR bands. The DNA products were then purified, sequenced in ABI PRISM® (Applied Biosystems), and finally compared with human mRNA sequences in the NCBI GenBank.

Adhesion and proliferation assays

For the adhesion and proliferation experiments, different non-specific or basement membrane extracellular matrix protein substrates were used. Gelatin (Gel; 1%) and bovine serum albumin (BSA; 10 µg/ml) or specific extracellular matrices like type I collagen from rat tail (Col I; 20 µg/ml), human fibronectin (FN; 10 µg/ml), laminin from murine sarcoma (LN; 10 µg/ml) and type IV collagen from human placenta (Col IV; 50 µg/ml) were used to coat tissue culture plates. Additionally, attempts were made to simulate basal membranes using different combinations of joint matrices with LN (4 µg/ml) and Col IV (50 µg/ml). All proteins were purchased from Sigma-Aldrich except type I collagen, which was purchased from Upstate Millipore (Billerica, MA, USA). Each

protein was reconstituted according to the manufacturer's recommendations. Matrix component solutions in culture medium were applied in 96-well culture plates and maintained for 2 hours at 37°C in a humidified atmosphere containing 5% CO_2. Coating solutions were then aspired and wells were briefly washed with PBS before cell plating. Cells were plated at densities between 3 – 15 x 10^4 cells/cm^2.

Adhesion experiments were conducted according to the Crystal Violet Dye Elution procedure[28]. In brief, after 24 hours of culture, cells were fixed with 3% paraformaldehyde solution for 30 minutes, washed thoroughly in distilled H_2O, and then stained with an aqueous solution of 0.25% crystal violet for 20 minutes. After successive washes, the dye was eluted for 30 minutes in 33% acetic acid. The absorbance was measured using an ELISA reader (ELx800, Bio-Tek Instruments Inc., USA) at 590 nm. Three independent experiments were carried out in triplicate.

Cell proliferation experiments were performed at 24 and 72 hours by cellular uptake of bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU). Cells were pulsed with 10 µM BrdU for 3 hours. The protocol was carried out according to the manufacturers' instructions (Cell Proliferation ELISA BrdU colorimetric; Roche Applied Science, Mannheim, Germany). The absorbance was measured at 370 nm with a reference wavelength of 492 nm. Three independent experiments were carried out in quadruplicate.

Data analysis

For the cellular adhesion and proliferation experiments, all data are presented as means ± SD. The means were analyzed using analysis of variance for *Dunnett's Multiple*

Comparison Test (GraphPad Prism version 4.0, GraphPad Software, San Diego, California, USA). Differences were considered statistically significant when *p* values were less than 0.05.

Results

Isolation and expansion of primary cell cultures (ADS and LSC)

Two or three days after being seeded onto an MMC-inactivated 3T3-SA feeder layer, colonies of LSC cells were observed by phase contrast microscopy. Cells in colonies were very small, tightly arranged and surrounded by 3T3 fibroblasts. LSC exhibited a high nucleus-to-cytoplasm ratio. Five or seven days later, colonies of cells appeared to be spherical (Figure 1a), which indicates clonal proliferation. ADS cells had fibroblast-like morphology (Figure 1b). Once they had adhered, they proliferated homogenously, with doubling times of 7 to 10 days.

Differentiation into adipogenic and osteogenic cells

To ensure that we were using ADS cells capable of multipotent lineage differentiation and plasticity, pool of cells were differentiated into adipogenic and osteogenic (Figure 2) lineages. Optimal differentiation times were between 21 and 28 days in an induction medium. This confirms the differentiation potential of ADS cells.

Flow cytometry and immunocytochemistry characterization

The expression of specific hematopoietic and/or mesenchymal progenitor cell markers, such as CD34 and CD90, was determined by flow cytometry. We also characterized the ABCG2 transporter cassette, which is considered a side population stem cell and putative LSC marker. ADS cells expressed CD34 (14.6%) and CD90 (94.3%) markers (not shown). These data confirm that lipoaspirate-derived cells have a mesenchymal progenitor origin. LSC cultures were practically negative for both CD34 and ABCG2

markers, and presented a very low expression for CD90 (7.4%). Markers for stratified epithelia, such as basic and acid keratins 1, 5, 10 and 14 (CK1/5/10/14), and for differentiated epithelia, such as keratins 3 and 76 (CK3/76), were determined by immunocytochemistry approaches (Figure 3) using a set of cytokeratin-specific antibodies. Surprisingly, ADS were moderately positive for CK3/76 (Figure 3e). This indicates that there could be a subpopulation in these cultures, which has intrinsic potential for epithelial profiles. LSC were also slightly positive for this marker, which suggests that there was some degree of differentiation in cell cultures or that cells with undifferentiated and differentiated characteristics were found in the isolation procedure. Furthermore, LSC were slightly positive for CK1/5/10/14 markers (Figure 3c). ADS cells were negative for CK1/5/10/14.

Western blot characterization

We carried out Western blot experiments to better characterize and confirm the expression of potential markers in adult ADS cells and to compare the expression profile of human LSC. Extracts from normal corneal epithelial cells were used as a control for the protein profile. Expression of CK3/76, CK1/5/10/14 and CK12 was analyzed. In these experiments, we also included transcription factor p63, which is used as a marker for corneoscleral limbal progenitor cells, and cytokeratin 19 (CK19), which is a component of intermediate filaments localized in the basal layer of corneoscleral limbus epithelia. The p63 expression was very high in LSC extracts (Figure 4), which confirms that this protein is a good specific marker for this cell line. In accordance with immunocytochemistry analyses (Figure 3), the presence of cytokeratins could be

confirmed in LSC and adult ADS cell extracts. LSC expressed high amounts of all cytokeratins in the assayed panel, which indicates that there were some differentiated cells in the culture. ADS cells exhibited a moderate amount of CK3/76, associated with weak expression of CK1/5/10/14 (Figure 4). The latter was exclusively observed by the presence of 68 and 50 KDa bands in autoradiography films.

Expression of ABCG2, p63 and cytokeratins mRNA

LSC can express a small amount of ABCG2 transport cassette, due to an existing side population with progenitor characteristics, as observed in previous studies[29,30]. Accordingly, the expression of ABCG2 mRNA was detected in LSC cells (Figure 5). We also identified the expression of ABCG2 mRNA in the adult ADS cell population (Figure 5). Moreover, the expression of ABCG2 mRNA decreased ~8-fold over time, after differential adhesion in culture. This indicates that there was some degree of cell differentiation *in vitro*. The p63α mRNA expression was elevated in LSC, which confirms this protein as a specific marker for progenitor cells from human corneal limbus epithelia. Interestingly, the adult ADS cell population can also express low amounts of mRNA for the α-isoform of this transcription factor, and these mRNA levels remained constant during the cell culture. Our data showed p63 mRNA content in ADS cells 10 cycles behind LSC cells (Figure 5). This means that there was more than a 100-fold difference in mRNA. This difference could easily mask the presence of p63 protein in ADS cells when analyzed by Western blot (Figure 4). Expression of mRNA for CK3 and CK12 was elevated in LSC, in accordance with previous results (Figure 3 and 4). We could not identify CK3 mRNA expression in ADS cells, but we noted weak expression

for CK12 and CK76, which indicates that a certain epithelial profile was intrinsic or acquired over time in adhesion culture selection. Taken together, our results suggest that ADS cells could be involved in epithelial-like differentiation.

Cytokeratin 12 expression in ADS cells

To confirm the expression of cytokeratins in ADS cells, we carried out PCR analysis from total RNA reverse transcribed using specific primers for CK3 (KRT3) and CK12 (KRT12). Amplified DNAs of the expected size (0.2 Kb) were identified for both markers in normal human corneal epithelial cells and LSC. CK12 was positive in ADS cells (Figure 6). The DNA product obtained from ADS was sequenced (ABI PRISM®), compared (NCBI GenBank, Reference Sequence: NM_000223.3) and its specificity was confirmed (83.2% - 93%) for both forward and reverse CK12 mRNA human sequence (Figure 6).

Differential adhesive and proliferative characteristics

Because the niche is a crucial microenvironment for the maintenance of progenitor cell growth and differentiation, we hypothesized that certain *in vitro* substrates could promote adhesive and/or proliferative differential behavior for ADS cells. Therefore, we carried out cell-adhesion and proliferation assays using different basement membrane extracellular matrix proteins and combinations of them. ADS cells changed their proliferative state and adhesion behavior in several of the matrices that were assayed. All of the tested specific protein matrices caused improved cellular adhesion with a significant increase in adhesion on FN, Col I and Col IV at 24 hours (Figure 7). However,

the proliferative state of ADS cells on LN substrates, was impaired, as a decrease in BrdU uptake was found between 24 and 72 hours on this protein matrix. These results indicate that the extracellular matrix environments play a determining role, with differential behavior of the ADS population on LN substrates (Figure 7).

Discussion

To date, white adipose tissue is the most abundant source of cells and the most readily available. ADS cells obtained from human lipoaspirates and bone marrow have an excellent capacity to differentiate toward several cell types[14,16-22]. However, the potential of ADS cells to differentiate into cells with epithelial characteristics is still largely unknown.

The absence of a specific panel of markers for LSC hampers cell identification and selection for differentiation and characterization studies of epithelial origin in the cornea. As expected, flow cytometry revealed that the stem cell markers CD34 and CD90 were negative for LSC. However, ADS cells expressed these markers, which were characteristic of undifferentiated progenitor cells of mesenchymal origin.

As mentioned, transcription factor p63 is a member of the p53 family. Its presence in the cell population of the basal layer of the epithelial limbic region may be indicative of the cells' high proliferative potential[31]. Western blot analysis revealed high expression of this nuclear protein using mAbs 4A4, which recognizes all p63 isoforms expressed in the corneal limbic cells. This protein is highly expressed in the basal cells of some human epithelial tissues, and the dominant negative truncated α-isoform ΔNp63 is predominant in these populations[32,33]. By qRT-PCR, ΔNp63α mRNA was strongly expressed in LSC. Moreover, this transcription factor was expressed at a low level in lipoaspirate subpopulations throughout the time in culture. Our findings are consistent with previous studies[8], which support the hypothesis that ΔNp63α is likely to identify the proliferative progenitor cell population from the eye limbus. Moreover, the expression of this protein in human ADS cell types allows us to identify another potential cell characteristic in

these populations that have highly proliferative features in *ex-vivo* expansion and cell therapy applications.

We also used different techniques to study the expression of the ATP-binding cassette transporter protein ABCG2. This marker has recently been proposed for the identification of a wide variety of progenitor cells and it is believed to be a molecular determinant of the side population phenotype when it is associated with the bis-benzimide nuclear staining method[34]. Previous reports[29,30] demonstrated that ABCG2 protein is located in the plasma cell membrane and cytoplasm of some cells in the basal layer of corneal epithelium. ABCG2 expression is thought to be a common attribute of stem cells that protects them against drugs and toxins[6]. However, the validity of ABCG2 as a marker of LSC lineage remains to be clarified. Expression analysis of ABCG2 mRNA indicated a small amount of this surface protein in the LSC population. Interestingly, the quantity was slightly higher in ADS cells. These data indicate the presence of a putative side population phenotype with intrinsic potential for proliferation and self-renewal, as previously described for limbic epithelial cells[29]. This finding still needs to be confirmed for ADS cells and we are carrying out further experiments to better characterize this behavior.

We also evaluated the capability of ADS cells to express a set of undifferentiated and differentiated epithelial keratins. Keratin filaments are one of the first epithelial-specific structural proteins to be synthesized in a differentiation program[35]. The presence of CK3/76 has been considered a characteristic of differentiation for human cornea epithelial cells, and could be employed to distinguish these from epithelial cells from the basal layer of the corneal limbic area[6,31,36,37]. Surprisingly, immunofluorescence and

Western blot approaches revealed positive staining using specific CK3/76 mAbs in ADS cells. In addition, our analysis by qRT-PCR revealed constant expression of CK76 and a progressive increase in CK12 after differential cell adhesion in culture. Specificity for human CK12 was confirmed by DNA sequencing. Thus, our data indicated that the antibodies against CK3/76 could identify both CKs in LSC and corneal epithelium, but only the expression of CK76 in ADS cells.

Finally, we wanted to establish the behavior of the ADS cells in several matrices, to observe the attachment and proliferation characteristics affected by factors related to the cells' environment. We found that ADS cells exhibited improved and homogeneous behavior for adhesion and proliferation on the proteins of extracellular matrices encountered in basal membranes.

In summary, the data obtained from this study suggest that ADS cells could be of potential application on the human ocular surface. The expression of stem cell progeny markers and the progressive, spontaneous increase in cytokeratin expression by cell adhesion selection in cell cultures indicates that these cells have the potential and capability to acquire epithelial-like characteristics in appropriate conditions. Recent studies have tried to differentiate human embryonic progenitor cells[38], adult bone-marrow mesenchymal cultures[39], adult progenitor cells from epidermis[40], hair follicle[41] and dental pulp[42] stem cells into cells with epithelial features. We are confident that the use of a suitable cell environment and conditioned media, supplemented with appropriate growth factors, could induce the differentiation of ADS cells from human lipoaspirates into cells with epithelial characteristics.

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FIGURES LEGENDS

Figure 1. Primary culture by phase contrast microscopy. Human LSC on 3T3-SA mitomycin-inactivated feeder layer exhibited spherical-shaped clonal colonies (a). Human lipoaspirate ADS cells presented fibroblast-like morphology after isolation and differential adhesion (b).

Figure 2. Adipogenic and osteogenic differentiation. Oil Red O positive staining for intracellular fat droplets (b and c) indicated that ADS cells were capable of adipogenic differentiation. Alizarin Red staining (d) and an elution assay confirmed better osteogenic differentiation after 28 days culture in induction medium. At this stage, alkaline phosphatase activity increased significantly when compared to control cultured ADS cells (a). Phase contrast microscopy (a, b and d); epifluorescence microscopy (c) with nuclear staining carried out with bis-benzimide (blue).

Figure 3. Indirect immunofluorescence for cytokeratins (CK). ADS cells were positive for CK3/76 (e), which is specific for differentiated epithelia. LSC were moderate positive for CK3/76 (b) and CK1/5/10/14 (c), which is specific for stratified epithelia. Control conditions (a and d). Nuclear staining was performed with bis-benzimide (blue). Bar = 20 µm.

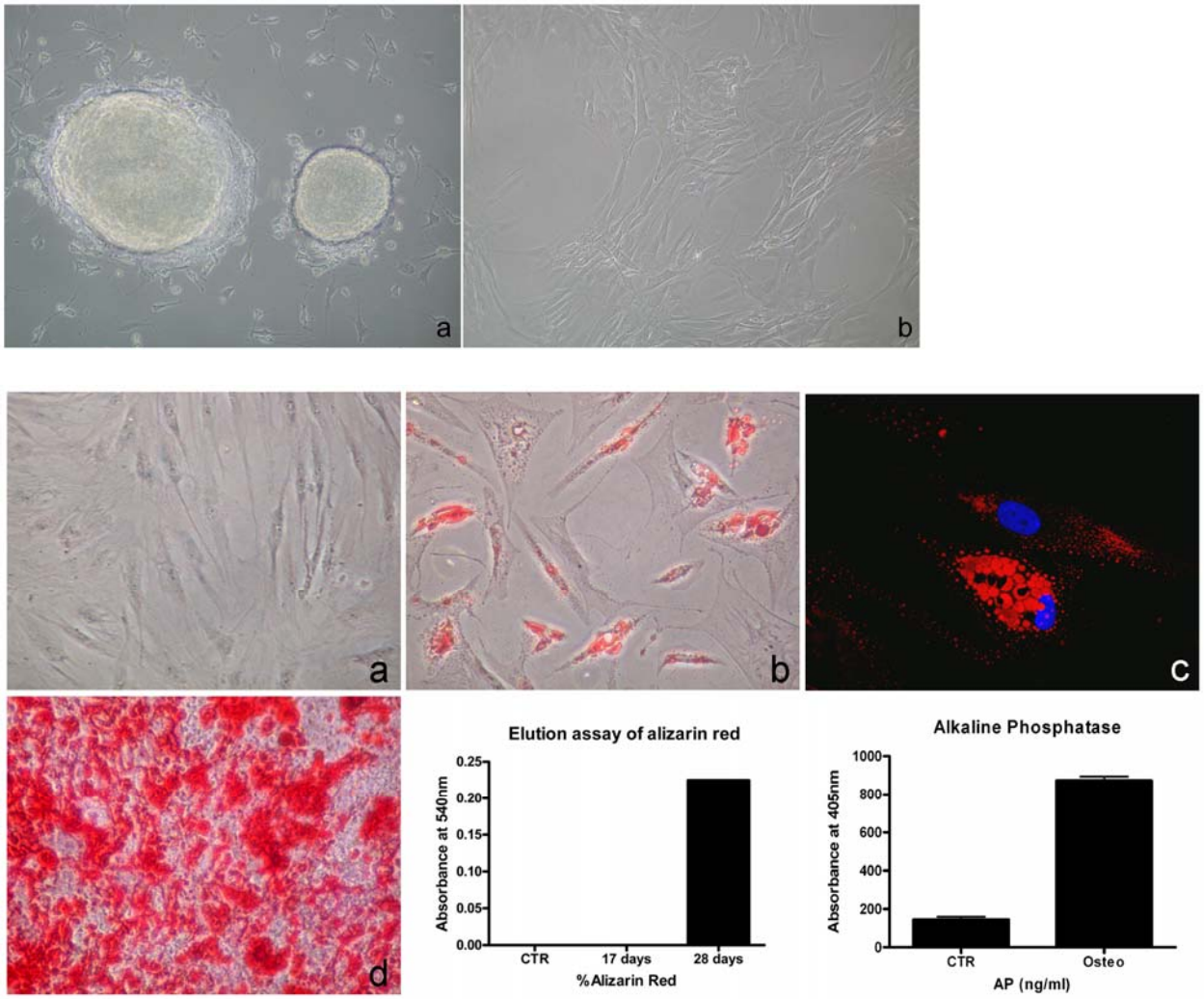
Figure 4. Western blot analysis for cytokeratins (CK) and p63 isoforms. LSC strongly expressed all isoforms for p63 and a wide panel of several cellular subsets of cytoketarins (CK) for differentiated epithelia. ADS cells were negative for p63, with weak

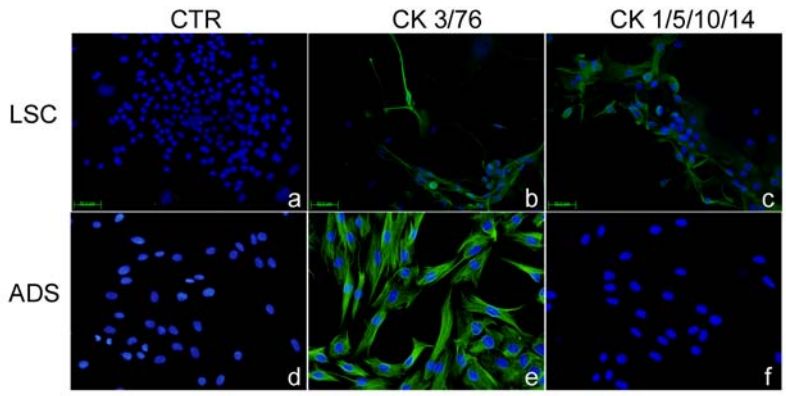
expression for CK3/76 and CK12. Normal human corneal epithelial cell (CO) extracts were used as a control for the expression profile. SDS-PAGE experiments were carried out with a two-fold concentration of ADS cell protein extract.

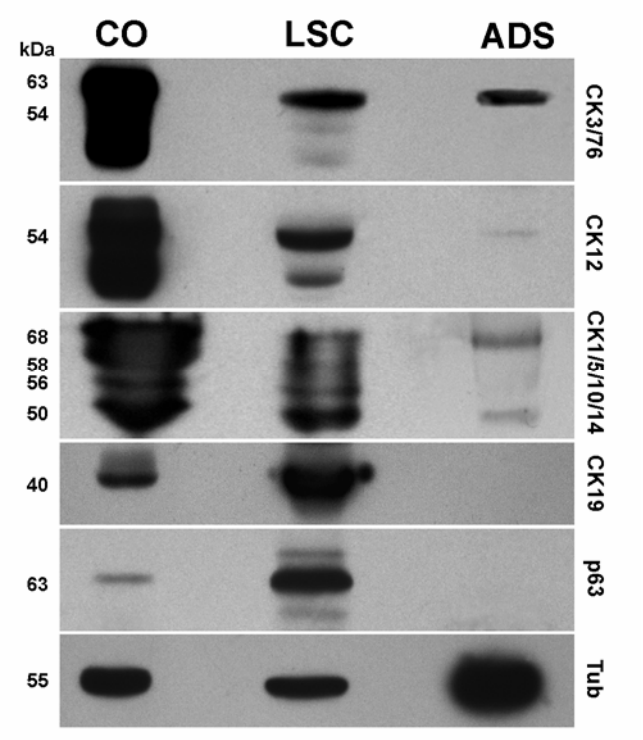
Figure 5. Expression of mRNA by qRT-PCR. Expression of ABCG2, the α-isoform of ΔNp63 and specific corneal epithelial marker CK3 (KRT3), CK12 (KRT12) and CK76 (KRT76) were quantified in normal human corneal epithelial cells (CO), LSC and ADS cells immediately isolated (ADS) and after differential adhesion (ADS1). ADS expressed mRNA for ABCG2 and p63α, but not for CK3. CK12 mRNA expression in ADS cells increased after differential adhesion in culture, while CK76 mRNA expression was maintained. Expression of mRNA is represented by the threshold cycle (Ct) ± SD and normalized for sample loading with 18S rRNA. Note that an increase of 2 in Ct indicates approximately a four-fold decrease in mRNA expression. The number of qPCR cycles (± SD) obtained is represented in an associated table for each condition.

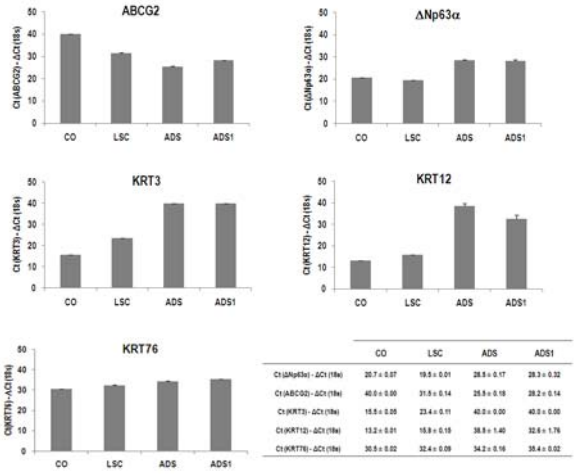
Figure 6. PCR confirmation for cytokeratins. The presence of CK12 (KRT12) in ADS cells was confirmed by PCR amplification of DNA obtained from reverse transcribed total RNA. The products were purified, sequenced and compared with the human CK12 mRNA sequence (mRNA CK12) that exhibited high specificity (≅ 90%). CK3 (KRT3) was absent in ADS cells. Normal human corneal epithelial cells (CO) and LSC were used as positive controls. KRT12f: the forward PCR product for CK12.

Figure 7. Adhesion and proliferation assays on extracellular matrix proteins. ADS cells behaved homogenously with improved adhesion (a; 24 hours) and proliferation (b and c; 24 and 72 hours respectively) on specific extracellular matrix proteins. LN substrates suggested that there was differential behavior between cellular adhesion and proliferation. CTR, control; GEL, gelatin 1%; BSA, bovine serum albumin 1%; Col I, type I collagen 20 µg/ml; FN, fibronectin 10 µg/ml; LN, laminin 10 µg/ml; A, Col IV, type IV collagen 50 µg/ml; Col IV + LN, type IV collagen 50 µg/ml and LN 4 µg/ml. Asterisk: *, $p < 0.05$; **, $p < 0.01$ with respect to the control.

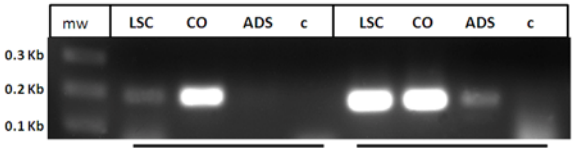



CTR
CK 3/76
CK 1/5/10/14
LSC
ADS
a
b
c
d
e
f


CO
LSC
ADS
kDa
63
54
CK3/76
54
CK12
68
58
56
50
CK1/5/10/14
40
CK19
63
p63
55
Tub



	CO	LSC	ADS	ADS1
Ct (ΔNp63α) - ΔCt (18s)	20.7 ± 0.07	19.5 ± 0.01	28.8 ± 0.17	28.3 ± 0.32
Ct (ABCG2) - ΔCt (18s)	40.0 ± 0.00	31.5 ± 0.14	25.5 ± 0.18	28.2 ± 0.14
Ct (KRT3) - ΔCt (18s)	15.5 ± 0.08	23.4 ± 0.11	40.0 ± 0.00	40.0 ± 0.00
Ct (KRT12) - ΔCt (18s)	13.2 ± 0.01	15.8 ± 0.18	38.8 ± 1.40	32.6 ± 1.76
Ct (KRT76) - ΔCt (18s)	30.5 ± 0.02	32.4 ± 0.09	34.2 ± 0.16	35.4 ± 0.02



KRT3 **KRT12**

```
                0 ------------------------------------------------------  0
              451 GTGCGAGCTCTAGAAGAGGCTAATACTGAGCTAGAAAATAAAATTCGAGA  500
KRT12f          1 ---------------------------------GGAGTCACT-T-TG--T  13
                                                    .|..||||. . .| |
mRNA CK12     501 ATGGTATGAAACACGAGGAACTGGGACTGCAGATGCTTCACAGAGCGATT  550
KRT12f         14 TCTG----------TC-C--AGTG-AGACCTC-GGAATAAGATCaTTTCa  48
                  .|.|         .| |  |.|| ||||||| |||||||||||||||||
mRNA CK12     551 ACAGCAAATATTATCCACTGATTGAAGACCTCAGGAATAAGATCATTTCA  600
KRT12f         49 GCCaGCATTGGAAATGCCCAGCTCCTCTTGCAGATTGACAATGCGAGACT  98
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
mRNA CK12     601 GCCAGCATTGGAAATGCCCAGCTCCTCTTGCAGATTGACAATGCGAGACT  650
KRT12f         99 AGCTGCTGAGGACTTCAGGATGAAGTATGAGAATGAACTGGCCCTG----  144
                  ||||||||||||||||||||||||||||||||||||||||||||||||
mRNA CK12     651 AGCTGCTGAGGACTTCAGGATGAAGTATGAGAATGAACTGGCCCTGCGCC  700
              144 ------------------------------------------------------  144
              701 AGGGCGTAGAGGCCGACATCAATGGCCTGCGCCGGGTGCTGGACGAGCTG  750
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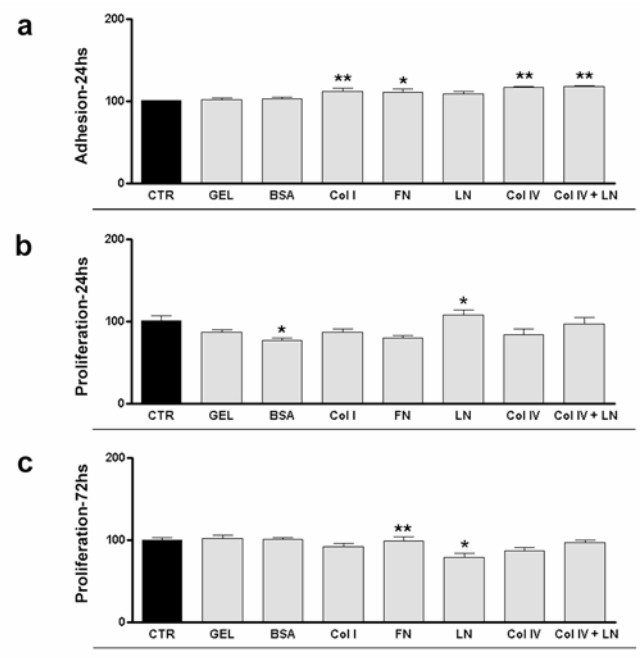
a
Adhesion-24hs
200
100
0
**
*
**
**
CTR
GEL
BSA
Col I
FN
LN
Col IV
Col IV + LN
b
Proliferation-24hs
200
100
0
*
*
CTR
GEL
BSA
Col I
FN
LN
Col IV
Col IV + LN
c
Proliferation-72hs
200
100
0
**
*
CTR
GEL
BSA
Col I
FN
LN
Col IV
Col IV + LN