In Vitro Simulation of Corneal Epithelium Microenvironment Induces a Corneal Epithelial-like Cell Phenotype from Human Adipose Tissue Mesenchymal Stem Cells

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ABSTRACT

PURPOSE: Transplantation of autologous corneal stem cells in not possible in cases of bilateral limbal stem cell deficiency (LSCD). To restore the ocular surface in these patients, an autologous extraocular source of stem cells is desirable to avoid dependence on deceased donor tissue and host immunosuppression of allogenic transplants. While bone marrow-derived mesenchymal stem cells (MSCs) can acquire certain characteristics of corneal epithelial cells, subcutaneous adipose tissue (AT) is more readily available and accessible. The aim of this study was to determine if extraocular human AT-derived MSCs (hAT-MSCs) can acquire *in vitro* some features of corneal epithelial-like cells.

METHODS: hAT-MSCs were isolated from human lipoaspirates and expanded up to 3-4 passages. We studied the immunophenotype of MSCs and demonstrated its multipotent capacity to differentiate towards osteoblasts, adipocytes, and chondrocytes. To test the capacity of differentiation of hAT-MSCs towards corneal epithelial-like cells, hAT-MSCs were cultured on substrata of plastic or collagen IV. We used basal culture medium (BM), BM conditioned with human corneal epithelial cells (HCEcBM), and BM conditioned with limbal fibroblasts (LFcBM).

RESULTS: The hAT-MSCs incubated for 15 days with HCEcBM acquired more polygonal and complex morphology as evaluated by phase-contrast microscopy and flow cytometry. Additionally, the expression of transforming growth factor-β receptor CD105 and corneal epithelial marker CK12 got increased as evaluated by flow cytometry, real-time reverse-transcription polymerase chain reaction, western-blot, and immunostaining. These changes were absent in hAT-MSCs incubated with unconditioned BM or with LFcBM.

CONCLUSIONS: Corneal epithelial-like cells can be induced from extraocular hAT-MSCs by subjecting them to an *in vitro* microenvironment containing conditioning signals derived from differentiated human corneal epithelial cells. Our results suggest that hAT-MSCs could provide a novel source of stem cells that hold the potential to restore sight lost in patients suffering from bilateral ocular surface failure due to LSCD.

KEYWORDS: mesenchymal stem cells, adipose-derived stem cells, cornea, epithelium, limbal stem cells, limbal stem cell deficiency

INTRODUCTION

Corneal transparency and visual function are dependent upon the integrity of the outermost epithelial layer that is maintained and continuously renewed by a population of stem cells (SCs). These SCs are located in the basal epithelium of the circular transitional zone between the cornea and conjunctiva-sclera known as the limbus.[1-4] The limbus provides a unique local microenvironmental niche in which corneal epithelial SCs, also called limbal epithelial SCs (LESCs), are surrounded by an extracellular matrix containing other non-stem cells. This microenvironment provides the cues or signals required to maintain the LESC phenotype.[3-5] Dysfunction of LESCs or of the limbal niche can occur as a consequence of a multitude of conditions, e.g., chemical or thermal burns, radiation, cicatrizing-autoimmune pathologies, trauma, infections etc. This usually results in chronic pain, persistent ocular surface inflammation, corneal opacity, and blindness.[6,7]

In cases of unilateral ocular trauma or disease, transplantation of autologous LESCs extracted from the limbal niche of the contralateral, unaffected healthy eye and expanded *in vitro* is the technique of choice.[8-11] In case of bilateral disease, which is actually more frequent, the recovery of a limbal biopsy is not possible due to the lack of healthy tissue. The use of deceased donor tissue in these cases transforms an autologous transplant into an allogenic one. To avoid immune rejection, allogenic transplantations require long-term systemic immunosuppression that subsequently increases the potential morbidity and health costs.[12] The ideal solution for bilateral diseases would be to maintain transplants of autologous origin by using an extraocular source of SCs.

In recent years, mesenchymal SCs (MSCs) have emerged as a major form of cell therapy. MSCs are pluripotent and, under the appropriate conditions, have the capacity to differentiate into mesodermal and non-mesodermal cell lineages.[13-15] While bone

marrow MSCs can acquire a corneal epithelial-like cell phenotype *in vitro* and *in vivo,*[16-20] the potential of extraocular adipose tissue (AT)-derived MSCs (AT-MSCs) for corneal epithelial regeneration[21,22] has not been fully explored. Subcutaneous AT is readily available and accessible and can be harvested by a simple and minimally invasive method. Furthermore, AT-MSCs are easy to isolate and culture, have a high expansion capacity, and can differentiate into epithelial cells.[13,23-27]

The microenvironment surrounding SCs produces triggering signals that direct them towards differentiation of specific cell phenotypes.[28] Thus, the purpose of this study was to analyze, by *in vitro* assays, the potential capacity of human AT-MSCs (hAT-MSCs) to acquire a corneal epithelial-like cell phenotype. We subjected hAT-MSCs to different *in vitro* microenvironments containing signals similar to those present in the *in vivo* corneal and limbal epithelium and determined the phenotypic outcomes.

MATERIALS AND METHODS

This study was approved by the IOBA Research Committee and by the Valladolid Medical School Ethics Committee. All procedures followed the guidelines of the Declaration of Helsinki for research involving human tissue.

Materials and Reagents

All reagents for cell culture were obtained from Invitrogen-Gibco (Inchinnan, UK) unless otherwise indicated. All reagents for flow cytometry were obtained from Beckman Coulter (Fullerton, CA, USA). Reagents used for RNA isolation and real-time reverse-transcription polymerase chain reaction (RT^2-PCR) were purchased from QIAGEN (RNeasy® Mini Kit and RNase-Free DNase Set, Hilden, Germany), from Invitrogen-Gibco (Quant-iT™ RNA assay Kit and SuperScript® VILO™ cDNA

Synthesis Kit), and from Applied Biosystem (Taqman® Universal Master Mix and Taqman® probes, Foster City, CA, USA). Reagents for western-blot analysis were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Isolation and Expansion of hAT-MSCs

Subcutaneous human AT from four different donors was obtained from cosmetic liposuction procedures after written informed consent was obtained. Cells were isolated using the procedure previously described by Zuk *et al.*[13] Lipoaspirates were digested at 37ºC for 30 minutes with 0.075% collagenase type I, incubated with 160 mM NH_4Cl erythrocyte lysing buffer, (Sigma-Aldrich, St. Louis, MO, USA) and filtered through 100 μm and 40 μm mesh filters. Until passage (P) 3-4, cells were cultured in basal medium (BM) composed of low-glucose Dulbecco's modified Eagle's medium (DMEM), 1 mM sodium pyruvate, 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37ºC, 5% CO_2 and 95% humidity. Under standard culture conditions the cells were cultured in BM and the passages were made at a density of 3,000-6,000 cells/cm^2 when cultures reached 90% of confluence.

Immunophenotypic Analysis

According to the International Society for Cellular Therapy (ISCT) position statement,[29] cell surface marker expression was performed by flow cytometry (Cytomics FC 500 Cytometer, Beckman Coulter). P3-P4 hAT-MSCs were harvested, washed and blocked with 40 μg/ml immunoglobulin G (Sigma-Aldrich) at room temperature (RT) for 15 minutes. Afterwards, cells were incubated with 10 μl anti-human monoclonal antibodies (Table 1) at 4ºC for 30 minutes in darkness. Alternatively, they were incubated with the corresponding mouse anti-human isotypic-matched controls IgG1-FITC, IgG1-PE,

IgG1-APC, IgG1-PC7 and IgG2a-FITC. For each assay, at least 5 x 10^4 cells were analyzed.

In vitro Multilineage Differentiation

For adipogenic and osteogenic differentiation, P3-P4 hAT-MSCs were cultured at a density of 10,000 cells/cm^2 using a StemPro Adipogenesis Differentiation Kit for 15 days and a Stem Pro Osteogenesis Differentiation Kit for 29 days following the manufacturer's instructions. To achieve chondroblast differentiation pellets, 10^6 hAT-MSCs were maintained in StemXVivo™ Chondrogenic Base Media with StemXVivo™ Chondrogenic Supplement (RD Systems, Minneapolis, MN, USA) for 27 days. Adipocyte and osteoblast differentiation was evidenced by Oil Red O and von Kossa staining, respectively. To confirm chondroblast differentiation, hAT-MSC pellets were fixed with paraformaldehyde, embedded in Optimal Cutting Temperature Compound (Aname, Madrid, Spain), sectioned, and stained with Alcian blue dye.

Isolation and Expansion of Human Limbal Fibroblasts

Human corneoscleral tissues from 10 different deceased donors were obtained from the Barraquer Eye Bank (Barcelona, Spain). Excessive sclera, conjunctiva, and iris were removed, and the central corneal button was extracted. The limbal epithelium was separated from the stroma by incubation with 5 mg/ml dispase II (Roche Diagnostics Corporation, Basel, Switzerland). Then the limbal stroma was digested with 2 mg/ml collagenase I overnight at 37ºC, and the resulting cells were cultured and expanded in BM plus 1% non-essential amino acids at 37ºC, 5% CO_2, and 95% humidity until P3.

Conditioning of Culture Media and Coating of Tissue Culture Plates with Collagen IV

Conditioning of hAT-MSC BM by human corneal epithelial (HCE) cells,[30] kindly provided by Arto Urtti (University of Helsinki, Finland),[31] was performed by culturing preconfluent HCE cells with 200 µl/cm^2 hAT-MSC BM for 24 hours. HCE-conditioned BM (HCEcBM) was collected daily for 4 consecutive days and finally pooled.
Conditioning of hAT-MSC medium by limbal fibroblasts (LFs) at P3-P4 was performed following the protocol previously described by Ahmad *et al.* with some modifications.[32] Mitotically inactivated LFs were cultured with 200 µl/cm^2 hAT-MSC BM, and the LF-conditioned BM (LFcBM) was collected daily for 7 days and finally pooled. Pools of harvested culture media were centrifuged at 277 g, filtered through a 0.22 µm pore to avoid any kind of cross contamination, and stored at -80ºC until used. Tissue culture plates were coated with collagen IV (Sigma-Aldrich) following the protocol previously described by Ahmad *et al.*[32] and following the manufacturer's instructions.
For the induction of corneal epithelial differentiation, P3-P4 hAT-MSCs from four different donors were plated separately at a density of 40,000 cells/cm^2 on polystyrene and collagen IV coated tissue culture plates. Epithelial differentiation was induced with 200 µl/cm^2 BM, HCEcBM, or LFcBM. Culture media were changed twice a week. Cell-viability and cell-cycle were studied by flow cytometry with the Annexin V-FITC Kit and Coulter® DNA Prep™ Reagents Kit, respectively, following the manufacturer's protocols. For each assay, at least 2 x 10^4 cells were analyzed.

RNA Isolation and RT^2-PCR

Total RNA was isolated from P3-P4 hAT-MSCs cultured under different differentiation conditions for 1, 8, 15, and 22 days using a previously described method.[33] Taqman Universal PCR Master Mix and specific Taqman® probes for cytokeratin 3 (*CK3,* Hs00365080_m1), cytokeratin 12 (*CK12,* Hs00165015_m1) and glyceraldehyde

phosphate dehydrogenase (*GAPDH,*4352934E) were used. Results were analyzed by the comparative "cycle-threshold method".[34] *GAPDH* was used as a housekeeping reporter gene, and hAT-MSCs grown in BM on plastic for each corresponding time point were used for calibration.

Western Blot

P3-P4 hAT-MSCs incubated for 15 days under differentiation-inducing conditions were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer plus protease and phosphatase inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 0.033 U/ml aprotinin, 1 mM sodium orthovanadate, Sigma-Aldrich). Homogenate proteins (20 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked in 5% dried milk and 4% FBS in Tris-buffered saline with 0.05% Tween-20 for 1 hour at RT and then incubated with the primary antibodies diluted in blocking buffer (Table 1) overnight at 4ºC. Membranes were incubated for 1 hour at RT with the secondary antibodies donkey anti-mouse-HRP 1:5000 (The Jackson Laboratory, Bar Harbor, ME, USA) or donkey anti-rabbit-HRP 1:2000 (Santa Cruz, Heidelberg, Germany). Protein extracts from HCE cells were used as positive controls. Immunoreactive bands were visualized by a chemiluminescence method using the ChemiDoc gel documentation system (Bio-Rad), and images were analyzed with the Quantity One software (Bio-Rad) using hAT-MSCs cultured with BM on plastic as the control.

Immunofluorescence

Cells were fixed in cold methanol and permeabilized with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (Sigma-Aldrich). After that, samples were incubated for 1 hour with 5% donkey serum (Sigma-Aldrich) in PBS at RT to block nonspecific binding and then at 4ºC overnight with cell-specific monoclonal antibodies (Table 1) diluted in blocking buffer. Afterwards, the cells were incubated for 1 hour at RT in darkness with corresponding secondary antibody (Alexa Fluor® 488 donkey anti-mouse IgG 1:200 or Alexa Fluor® 488 donkey anti-rabbit IgG 1:300, Invitrogen-Gibco) diluted in PBS. Finally, cell nuclei were counterstained with propidium iodide and visualized in an inverted fluorescence microscope (DM4000B Leica, Wetzlar, Germany). Primary antibodies were omitted in negative control studies.

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). Statistics were analyzed using the Statistical Procedures for the Social Sciences (SPSS 15.0, IBM Corporation, Somers, NY, USA). Statistical significance was determined by two-way factorial repeated measures analysis of variance (ANOVA) and analyzed by Bonferroni post hoc tests for multiple comparisons. Two-sided P-values ≤ 0.05 were considered statistically significant.

RESULTS

Characterization of Undifferentiated hAT-MSCs

Cultured stromal cells in P3-P4 isolated from lipoaspirates from four different donors fulfilled the minimal criteria for MSCs established by the ISCT.[30] In the analysis by flow cytometry (Fig. 1a), P3-P4 hAT-MSCs were positive for the surface markers

CD73, CD90, and CD105 (> 95% of the population) and were negative for CD14, CD19, CD34, CD45, and HLA-DR (< 2% of the population). When hAT-MSCs were cultured under adipogenic conditions, we observed a significant number of intracellular lipid droplets stained with Oil Red O (Fig. 1b). Under osteogenic conditions, calcium deposits, as shown by von Kossa staining, were present in the hAT-MSCs (Fig. 1b). Chondrogenic differentiation micromass culture of hAT-MSCs resulted in the formation of dense nodules and the appearance of sulphated proteoglycans in the extracellular matrix stained by Alcian Blue (Fig. 1b).

Characterization of HCE Cells and Human LFs

By inverted phase contrast microscopy, HCE cells had the typical polygonal morphology of epithelial cells (Fig. 1c). By immunofluorescence microscopy (Fig. 1c), the expressions of corneal epithelial specific CK3 and CK12 markers were positive, typical for HCE cells.[35,36]

Human LFs had an elongated, spindle-like morphology (Fig. 1d). By immunofluorescence, P3 LFs expressed the fibroblast markers vimentin, CD90, and FSP1.[37-39] They did not express pan-keratin, indicating that the cell culture was free of contamination by epithelial cells (Fig. 1d).

Effect of Differentiation Inducing Conditions on hAT-MSC Morphology, Viability, and Cell Cycle

Under standard conditions, hAT-MSCs had a spindle-like shape and low complexity. When hAT-MSCs were cultured to over-confluence with BM and LFcBM on plastic and collagen IV for 15 days, they also had a spindle-like shape as shown by phase contrast microscopy (Fig. 2). However, cells cultured with HCEcBM acquired a more polygonal, epithelial-like morphology (Fig. 2). Additionally, confluence decreased and

the cells acquired an epithelial-like growth in monolayers. Cell complexity was affected by the different culture conditions, as shown by flow cytometry. The highest increase in cell complexity occurred in cells incubated with HCEcBM (Fig. 2). These results were correlated with the increase in cytoplasmic granules observed by inverted phase contrast microscopy (Fig. 2). The substratum had no significant effect on cell complexity. hAT-MSC viability was around 95% in all culture conditions. Analysis of cell cycle showed that 75% of hAT-MSCs grown under standard conditions (in BM at 70-80% of confluence) were in phase G1/G0. This percentage increased significantly up to 85% when cells were cultured in the other non-standard conditions (data not shown).

Effect of Differentiation Inducing Conditions on hAT-MSC Immunophenotype

The expression of CD73, CD90, CD105, CD14, CD19, CD34, CD45, and HLA-DR markers was analyzed by flow cytometry in hAT-MSCs grown for 15 days under different conditions. The percentage of cells expressing the positive surface markers for hAT-MSCs CD73, CD90, CD105 (> 95% of the population) and the negative surface markers CD14, CD19, CD34, CD45, and HLA-DR (< 2% of the population) was not affected in cells grown under the different test conditions compared to hAT-MSCs cultured in standard conditions (in BM at 70-80% of confluence) (data not shown).

The mean fluorescence intensity (MFI) reflects the average amount of protein expressed per cell in absolute units. The MFI of the surface markers CD73, CD90, and CD105 increased under differentiation conditions when compared to standard conditions (Fig. 3). For CD105, the increase was significantly greater in cells grown in HCEcBM compared to cells grown in BM and LFcBM. There were no significant differences in the MFI of cells grown on plastic and collagen IV. The surface markers CD14, CD19, CD34, CD45, and HLA-DR did not change when compared to hAT-MSCs grown under standard conditions.

We also studied the expression of these markers in HCE cells and LFs. Surprisingly, HCE cells expressed CD73 and CD105 markers (82% and 67% of the population, respectively), but none of the others. Human LFs expressed CD73, CD90, and CD105 markers (97%, 95% and 86% of the population, respectively; data not shown). These were similar to the marker pattern shown by hAT-MSCs. However, MFIs for CD73, CD90, and CD105 in HCE cells and LFs were lower than in hAT-MSCs under any culture condition. Of these, the MFI for CD73 in HCE cells was significantly lower than in hAT-MSCs and LFs (data not shown).

Gene and Protein Expression Analysis of Corneal Epithelial Markers

To analyze how the different test conditions affected the expression of specific markers of corneal epithelial cells in hAT-MSCs, the expression of CK3 and CK12 was analyzed by RT^2-PCR, western blot, and immunofluorescence microscopy. There were no significant changes in relative mRNA expression of *CK3* over time for any of the culture conditions (Fig. 4). However, at day 22 the relative mRNA expression of *CK3* was significantly lower in hAT-MSCs incubated with LFcBM compared to BM. For hAT-MSCs grown on collagen IV, the decrease in *CK3* mRNA expression was significant on day 1 of culture for cells incubated with either HCEcBM or LFcBM compared to BM. Although the relative expression of *CK3* in cells grown over plastic was higher than in cells grown over collagen IV, the differences were not statistically significant (Fig. 4).

In the case of the CK12 marker, the relative *CK12* mRNA expression remained stable over time in hAT-MSCs incubated with LFcBM (Fig. 4) on both plastic and collagen IV substrata. In hAT-MSCs incubated with HCEcBM, the relative expression of *CK12* increased over time, reaching the highest point after 8 days of culture for both substrata. The differences were significant at 8 and 15 days in cells grown with HCEcBM on

plastic with respect to the same conditions at 1 day of culture. The relative increase of *CK12* mRNA expression in cells grown with HCEcBM on plastic for 8, 15, and 22 days was statistically significant with respect to cells cultured for these same periods with BM and LFcBM (Fig. 4). For cells grown on collagen IV, the relative *CK12* expression was significantly greater at 8 days for cells cultured with HCEcBM compared with LFcBM. Although the increase was higher in cells grown on plastic than in cells cultured on collagen IV, the difference was not significant (Fig. 4).

The expression of CK3 and CK12 protein was studied by western blot analysis and quantified by densitometry. Protein extracts from HCE cells were use as positive control for the identification of CK3 and CK12 specific bands (data not shown). At 15 days, hAT-MSC expression of CK3 was detected in a 64 KDa band under all growth conditions. For cells grown on collagen IV, the expression of CK3 significantly decreased with HCEcBM compared to those cultured with BM (Fig. 5). The expression of CK12 by hAT-MSCs was detected in a 108 KDa band under all growth conditions. For hAT-MSCs grown on plastic for 15 days, the expression of CK12 was significantly higher in cells incubated with HCEcBM compared with BM and LFcBM (Fig. 5). Although the expression of CK12 was higher in hAT-MSCs grown on plastic, the differences between substrata were not significant.

The expression of CK3 and CK12 proteins was also analyzed by immunofluorescence microscopy. The expression of some cytokeratins, such as CK12, CK3, and CK18, has been previously described in MSCs.[19,21,23,26,27] Consistent with this study, we saw that hAT-MSCs and LFs grown under standard conditions showed moderate expression of CK3 and CK12 (data not shown). The expression of CK3 remained in cells grown under all of the different conditions for 15 days, but was higher in cells grown on plastic than in cells grown on collagen IV (Fig. 6). However, CK12 was expressed with more

intensity in hAT-MSCs grown for 15 days under each condition. CK12 protein was present throughout the cytoplasm in HCE cells (Fig. 1c), LFs, and hAT-MSCs cultured under standard conditions (data not shown) and in BM (Fig. 6). However, in hAT-MSCs incubated with HCEcBM and LFcBM, CK12 was located in the perinuclear area (Fig. 6). There were no significant differences between plastic and collagen substrata in CK12 expression.

DISCUSSION

In the present study we tried to recreate some of the molecular signals and cues present in both the corneal and limbal microenvironments to analyze the *in vitro* potential of hAT-MSCs for acquiring a corneal epithelial-like cell phenotype. To that end, we cultured hAT-MSCs on plastic and collagen IV with hAT-MSC BM conditioned with supernatants collected from either corneal epithelial cells or limbal fibroblasts culture medium. Collagen IV is the main component of the limbal basement membrane, but it is much less predominant in the central portion of the cornea.[4,40-42] We used the combination of HCE supernatants together with the absence of collagen IV on the substratum to try to provide signals similar to those contained in the *in vivo* corneal epithelium microenvironment. We also attempted to recreate certain characteristic signals of the *in vivo* limbal niche microenvironment by using the combination of collagen IV as the substratum together with the presence of soluble factors secreted by LFs in the culture medium.[32,43,44]

None of the study conditions affected hAT-MSC viability. However, the hAT-MSC cell cycle was affected in cells subjected to the differentiation conditions. There was an increase of quiescent cells and a consequent decrease in the number of cells in division. This is consistent with the well-known observation that the differentiation process is accompanied by a decrease in cell proliferation.[45]

hAT-MSCs cultured with HCEcBM acquired epithelial-like growth in monolayers composed of cells with a more polygonal morphology. These cells also had higher complexity and an increase in the content of cytoplasmic granules. This is consistent with previous reports that described a high content of glycogen granules in corneal epithelial cells.[46] Thus, hAT-MSCs could acquire an epithelial-like cell phenotype when they are subjected to a microenvironment containing signals similar to those present in the corneal epithelium microenvironment.

In the present work we observed that HCE cells expressed the typical MSC markers CD73 and CD105. For the first time, we identified the expression of CD105, (endoglin, the type III receptor for transforming growth factor (TGF) –β)[47] in an epithelial cell lineage, suggesting that this molecule could mediate a different action of TGF-β in corneal epithelial cells.

The MFI of CD105 increased in cells incubated with HCEcBM while the corresponding CD73 and CD90 did not change significantly. In contrast with our findings, Ho *et al.* reported that MSCs derived from orbital fat and induced to differentiate into corneal epithelial cells decreased the expression of CD105.[48] These differences could be explained by the different sources of AT. The CD105 expression found in HCE cells, together with the increased MFI of CD105 in hAT-MSCs incubated with HCEcBM, suggest that in fact this protein could be a potential marker for corneal epithelial cell differentiation. In addition, the TGF-β secreted by fibroblasts and corneal epithelial cells has a paracrine or autocrine action, and it is implicated in the natural corneal wound healing process.[49]Altogether these data suggest that the morphological changes and the changes in expression of corneal epithelial markers observed in these cells could be mediated by factors such as TGF-β. This is consistent with the known involvement of TGF-β in differentiation processes such as mesenchymal-epithelial transition.[26,50,51]

We analyzed the expression of the well-established corneal epithelial markers CK3 and CK12 by RT^2-PCR, western blot, and immunofluorescence microscopy.[35,36] Both markers were expressed in low levels in hAT-MSCs and LFs grown under standard conditions. These data agree with previous studies in which CK3 and CK12 were expressed in rabbit bone marrow MSCs and in hAT-MSCs,[19,21] and some other cytokeratins such as CK18 were expressed in hAT-MSCs.[23,26,27]

Although the molecular weight of the protein CK12 is 54 KDa, we detected this protein by western blot in a band of 108 KDa. This indicates that the CK12 was in a homodimer conformation as reported for keratins that form intermediate filaments.[52]

The CK12 protein in hAT-MSCs cultured under standard conditions and grown with BM was expressed throughout the cytoplasm. However, in hAT-MSCs incubated with HCEcBM and LFcBM, the expression of CK12 was located in the perinuclear area. This change could be due to an increase in the synthesis of this protein in the endoplasmic reticulum,[53] as suggested by the greater amounts of CK12 seen in the western blots.

Our results showed that CK12 expression increased in hAT-MSCs grown with HCEcBM on plastic; however, the expression of CK3 did not significantly change. Ahmad *et al.*[32] obtained an increase of CK12 and CK3 mRNA expression when embryonic SCs were cultured with epithelial medium conditioned by LFs. In those experiments, the expression of CK12 occurred earlier than the peak expression of CK3. Perhaps if our incubation times had been longer we would have observed an incremental increase of CK3 mRNA levels as well. In addition, other research groups observed an increase in the expression of corneal epithelial markers and the acquisition of an epithelial-like phenotype in SCs incubated with medium conditioned by LFs.[32,44] In our experiments, we found that the expression of corneal epithelial markers in hAT-

MSCs grown with HCEcBM was higher than in cells incubated with LFcBM. Ahmad *et al.*[32] used embryonic SCs and Blazejewska *et al.*[44] used hair follicle SCs. These types of SCs seem, therefore, to behave differently than hAT-MSCs under limbal niche simulated conditions.

Studies carried out by Ho *et al.* showed that MSCs derived from subcutaneous AT had a lower capacity to differentiate into the corneal epithelial phenotype than those obtained from orbital fat.[48] Although their results are not consistent with ours, the differences could be explained by the fact that we cultured the cells for more days and at a higher level of confluence, which is a well-known important factor that modulates MSC differentiation.[54] The increase of CD105 and CK12 expression, together with the acquisition of a more epithelial-like morphology in hAT-MSCs grown with HCEcBM on plastic, indicate that hAT-MSCs subjected to signals similar to those contained in the corneal epithelium can acquire a corneal epithelial-like phenotype. In addition, several reports recently described the acquisition by bone marrow MSCs of a corneal epithelial-like phenotype when transplanted into animals with damaged corneas.[16,19,55-57]

Further investigations are needed to prove that hAT-MSCs can serve as a major source of SCs for corneal surface reconstruction. Nevertheless, we have provided preliminary evidence for the capacity of extraocular hAT-MSCs to acquire certain features of corneal epithelial-like cells by subjecting them to an *in vitro* microenvironment containing signals provided by differentiated corneal epithelial cells. *In vivo* experiments that consider the influence of the specific ocular surface microenvironment at the transplantation area will provide further insights about the real differentiation capacity of these SCs and their therapeutic potential.

CONCLUSIONS

Corneal epithelial-like cells can be induced from extraocular hAT-MSCs by subjecting them to an *in vitro* microenvironment containing conditioning signals derived from differentiated human corneal epithelial cells. Our results suggest that hAT-MSCs could provide a novel source of stem cells that hold the potential to restore sight lost in patients suffering from bilateral ocular surface failure due to limbal stem cell deficiency.

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DECLARATION OF INTERESTS

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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TABLE

Table 1. Primary antibodies used in flow cytometry, western blot, and immunofluorescence experiments.

Antibody	Specificity	Clone	Source	Technique and working concentration (µg/ml)
CD14-FITC	Monocytes and Macrophages	RM052	Beckman Coulter	FCM
CD19-PE	B cells	SJ25C1	BD Biosciences	FCM
CD34-PC7	Hematopoietic stem cells	581	Beckman Coulter	FCM
CD45-FITC	Pan-leukocyte marker	J33	Beckman Coulter	FCM
HLADR-FITC	Antigen-presenting cells	357	Beckman Coulter	FCM
CD73-PE	Mesenchymal stem cells	AD2	BD Biosciences	FCM
CD90-FITC	Mesenchymal stem cells	F15-42-1-5	Beckman Coulter	FCM
CD105-APC	Mesenchymal stem cells	2H6F11	Bioleyed	FCM
CK3	Corneal epithelial cells	AE-5	Mp Biomedicals	WB (2) IMF (20)
CK12	Corneal epithelial cells	H60	Santa Cruz Biotechnology	WB (1) IMF (4)
GAPDH	Housekeeping	10B8	Santa Cruz Biotechnology	WB (0.2)
Ab-1(CD-90)	Fibroblasts	AS02	EMD Biosciences	IMF (5)
FSP1	Fibroblasts	1B10	Abcam	IMF (2)
Vimentin	Fibroblasts	V9	Santa Cruz Biotechnology	IMF (0.4)
Pan-keratin	Epithelial cells	80	Abcam	IMF (5)

Beckman Coulter (Fullerton, CA, USA), BD Biosciences (San Jose, CA, USA), Bioleyend (San Diego, CA, USA), Mp Biomedicals (Illkirch, France), Santa Cruz Biotechnology (Heidelberg, Germany), EMD Biosciences (San Diego, CA, USA), Abcam (Cambridge, UK).

Abbreviations: FCM, flow cytometry; WB, western blot; IMF, immunofluorescence.

FIGURES

Figure 1

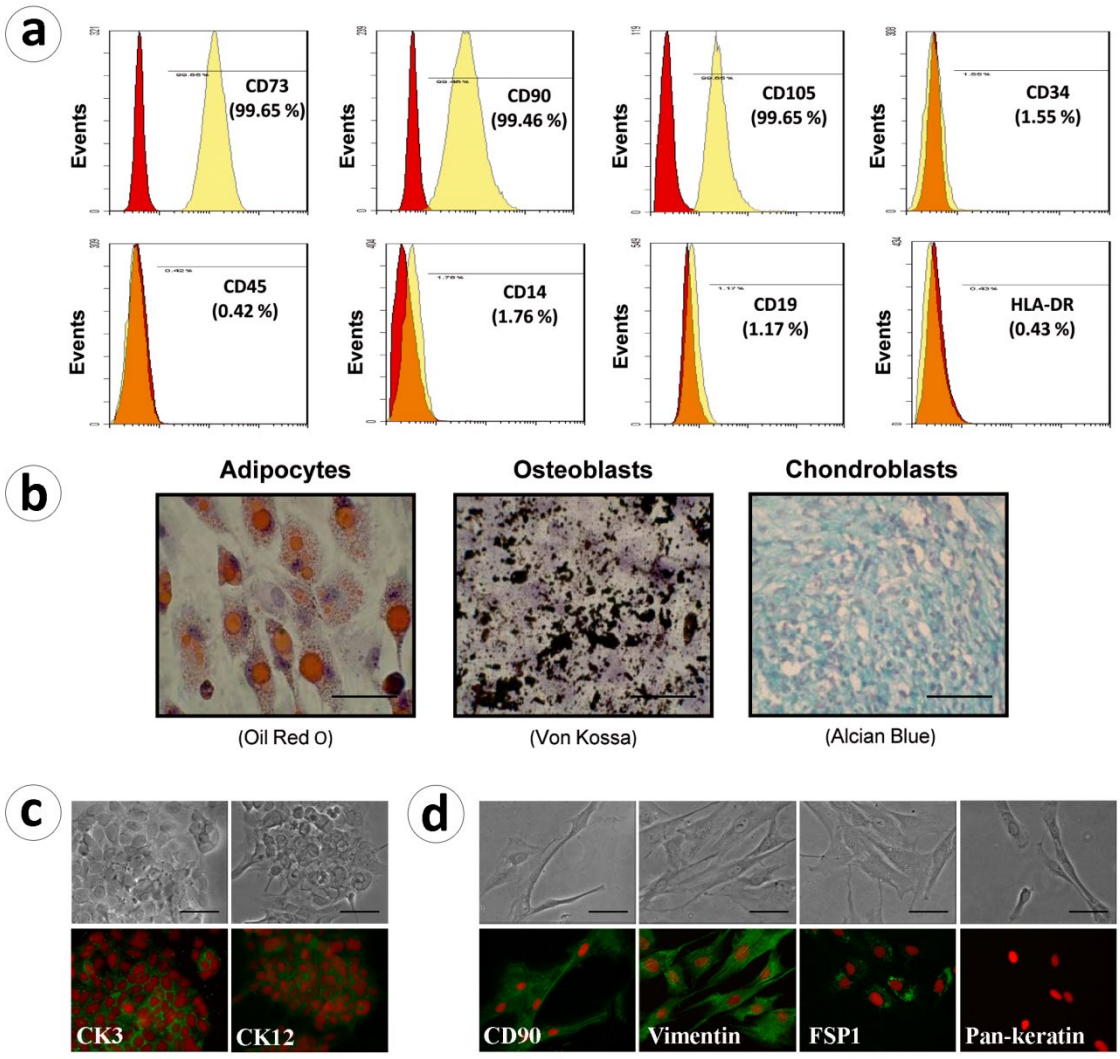

Figure 2

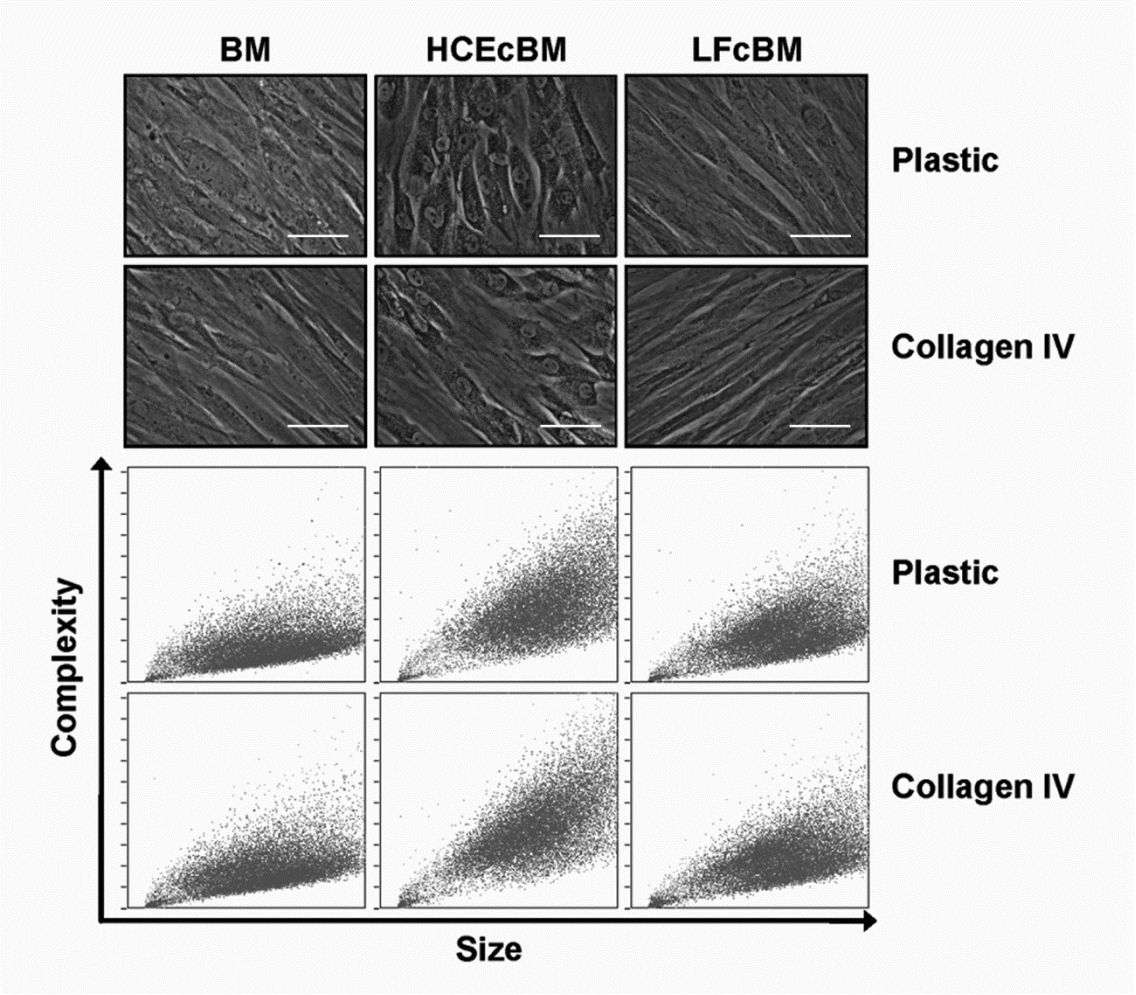
BM
HCEcBM
LFcBM
Plastic
Collagen IV
Plastic
Collagen IV
Complexity
Size

Figure 3

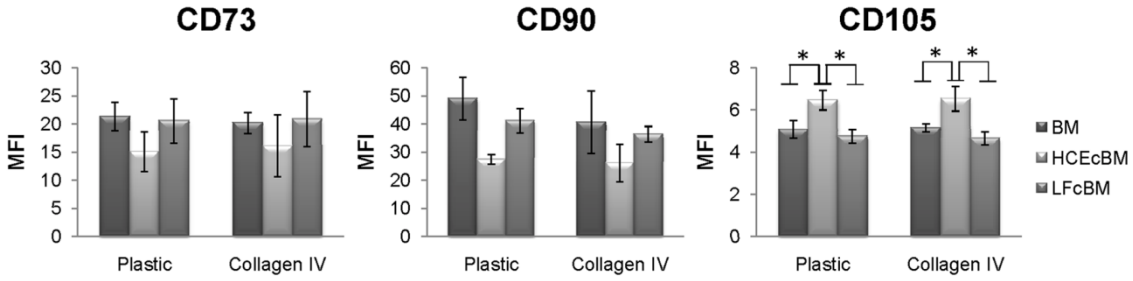
CD73
CD90
CD105
MFI
30
25
20
15
10
5
0
60
50
40
30
20
10
0
8
6
4
2
0
*
*
*
*
Plastic
Collagen IV
BM
HCEcBM
LFcBM

Figure 4

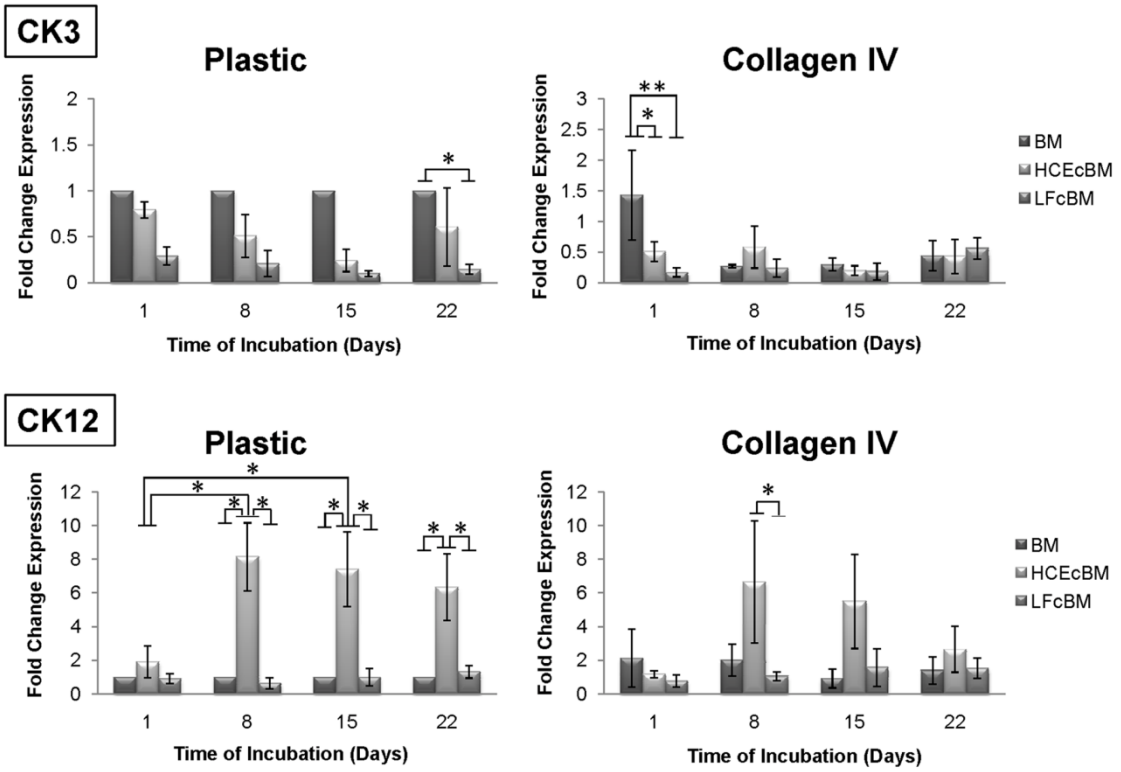
CK3
Plastic
Collagen IV
Fold Change Expression
Time of Incubation (Days)
BM
HCEcBM
LFcBM
CK12
Plastic
Collagen IV
Fold Change Expression
Time of Incubation (Days)
BM
HCEcBM
LFcBM

Figure 5

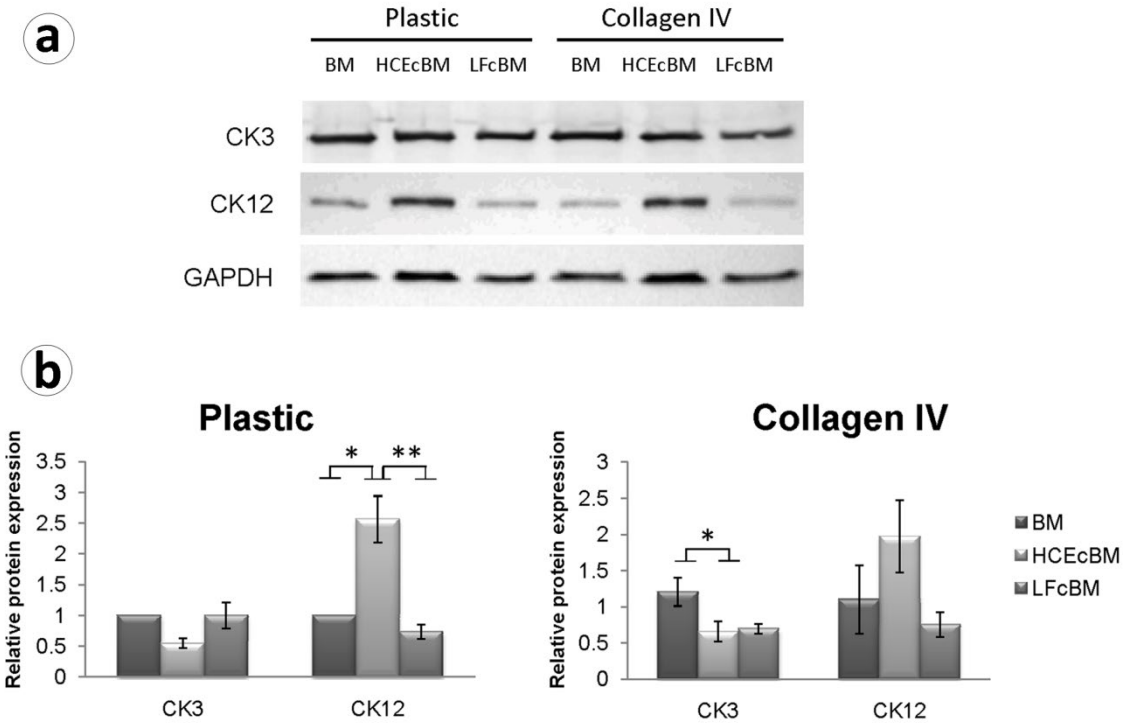
a
Plastic
Collagen IV
BM
HCEcBM
LFcBM
BM
HCEcBM
LFcBM
CK3
CK12
GAPDH
b
Plastic
Collagen IV
Relative protein expression
3.5
3
2.5
2
1.5
1
0.5
0
*
**
CK3
CK12
Relative protein expression
3
2.5
2
1.5
1
0.5
0
*
CK3
CK12
BM
HCEcBM
LFcBM

Figure 6

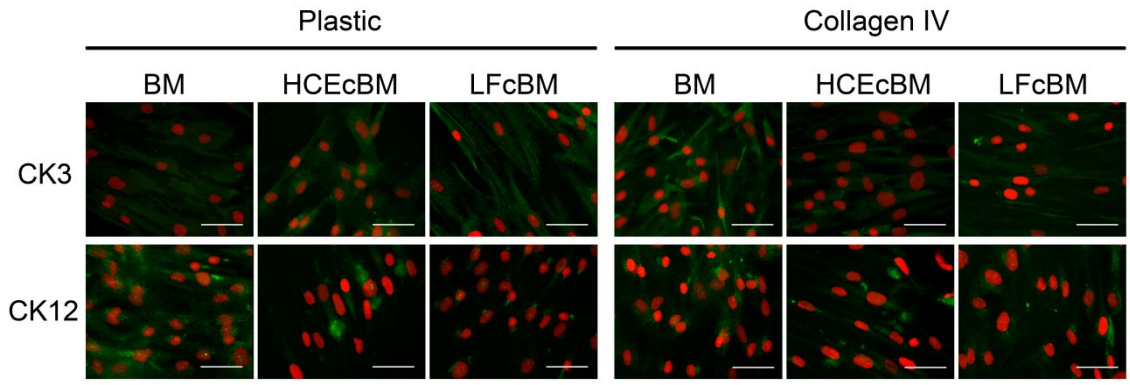
Plastic
Collagen IV
BM
HCEcBM
LFcBM
BM
HCEcBM
LFcBM
CK3
CK12

FIGURE CAPTIONS

Fig. 1 Characterization of hAT-MSCs, HCE cells, and LFs. (a) Flow cytometry analysis for the expression of the MSC markers in passage 3-4 hAT-MSCs grown under standard conditions. Representative of four independent experiments. Yellow histograms indicate the expression of markers, whereas the red histograms refer to the isotype controls. (b) Adipogenic, osteogenic, and chondrogenic differentiation of passage 3-4 hAT-MSCs after 15, 29 and 27 days of selective differentiation conditions respectively. Representative images of four independent experiments. (c) Immunoflourescence analysis of HCE cells for the expression of CK3 and CK12 (green fluorescence). Representative images of three independent experiments. (d) Immunoflourescence analysis of passage 3 LFs for the expression of CD90, vimentin, FSP1 and pan-keratin (green fluorescence). Representative images of three independent experiments. Nuclear staining was performed with propidium iodide (red). Scale bar: 50 μm

Fig. 2 Acquisition of epithelial-like cell morphology and increase of cell complexity in hAT-MSCs under different culture conditions. Bright phase contrast microphotographs and flow cytometry histograms of cell size and complexity of 40,000 hAT-MSCs/cm^2 in passage 3-4 cultured for 15 days in basal medium (BM), BM conditioned with human corneal epithelial (HCE) cells (HCEcBM), and BM conditioned with limbal fibroblasts (LFcBM) on plastic and collagen IV. hAT-MSCs cultured with HCEcBM acquired epithelial-like characteristics. Representative images of four independent experiments. Scale bar: 50 μm

Fig. 3 CD73, CD90, and CD105 mean fluorescence intensity (MFI) in hAT-MSCs under different culture conditions. The MFIs for CD73, CD90, and CD105, markers of undifferentiated hAT-MSCs, were analyzed by flow cytometry of 40,000 hAT-MSCs/cm^2. The cells were in passage 3-4 and cultured on plastic and collagen IV for 15 days in basal medium (BM), BM conditioned with human corneal epithelial (HCE) cells (HCEcBM), and BM conditioned with limbal fibroblasts (LFcBM). MFI is the average amount of protein expressed in absolute units per cell. Data represent the mean ± SEM from four independent experiments. Statistical significance was assessed by two-way factorial ANOVA. The MFI for CD105 was increased in hAT-MSCs cultured with HCEcBM. *$p<0.05$

Fig. 4 Relative expression of *CK3* and *CK12* mRNA in hAT-MSCs under different culture conditions.Relative mRNA expression of *CK3* and *CK12* was analyzed by RT^2-PCR of 40,000 hAT-MSCs/cm^2. The cells were in passage 3-4 and cultured on plastic and collagen IV in basal medium (BM), BM conditioned with human corneal epithelial **(**HCEcBM), and BM conditioned with limbal fibroblasts (LFcBM) at 1, 8, 15, and 22 days. Data represent the mean ± SEM from four independent experiments. Statistical significance was assessed by two-way factorial ANOVA. *CK12* mRNA expression in hAT-MSCs cultured with HCEcBM was significantly increased compared to the other media. *$p<0.05$, **$p<0.01$

Fig. 5 CK3 and CK12 protein expression in hAT-MSCs under different culture conditions. The protein expression of CK3 and CK12 was analyzed by immunoblot analysis of 40,000 hAT-MSCs/cm^2. The cells were in passage 3-4 and cultured on plastic and collagen IV for 15 days in basal medium (BM) and BM conditioned with human corneal epithelial cells (HCEcBM), and BM conditioned with limbal fibroblasts (LFcBM). (a) Representative immunoblots from three independent experiments. (b) Densitometry of three independent experiments. hAT-MSCs grown in BM on plastic were used as the control. Data were normalized by GAPDH and represent the mean ± SEM from three independent experiments. Statistical significance was assessed by two-way factorial ANOVA. CK12 protein expression in hAT-MSCs cultured with HCEcBM was significantly increased compared to the other media. *$p<0.05$, **$p<0.01$

Fig. 6 Analysis by immunofluorescence of CK3 and CK12 protein expression in hAT-MSCs under different culture conditions. Analysis of the CK3 and CK12 expression (green fluorescence) by immunofluorescence of 40,000 hAT-MSCs/cm^2. The cells were in passage 3-4 and cultured on plastic and collagen IV for 15 days in basal medium (BM), BM conditioned with human corneal epithelial cells (HCEcBM), and BM conditioned with limbal fibroblasts (LFcBM). Representative images of three independent experiments. Nuclear cell staining was performed with propidium iodide (red). CK12 protein expression was located in the perinuclear area of hAT-MSCs cultured in HCEcBM and LFcBM. Scale bar: 50 µm